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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**The Structure of Fitness Landscapes in Antibiotic Resistant Bacteria: Molecular
Origins and Evolutionary Consequences**

A dissertation submitted in partial satisfaction of the
requirements for the degree
Doctor of Philosophy

in

Physics

by

John Barrett Deris

Committee in charge:

Professor Terence Hwa, Chair
Professor Lin Chao
Professor Peter Geiduschek
Professor Alex Groisman
Professor Suckjoon Jun

2013

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Chair

University of California, San Diego

2013

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With permission from the coauthors, chapters 2-5 of this dissertation contain work from a manuscript that has been submitted for publication with the working title, "The innate growth bistability of antibiotic resistance," 2013, Deris, JB; Kim, M; Zhang, Z; Okano, H; Hermesen, R; Groisman, A; Hwa, T. The dissertation author and Minsu Kim are co-first authors in the submitted work.

Minsu Kim prepared all microfluidic results and methods used in this dissertation, and also constructed some strains for use in the microfluidic device. Zhongge Zhang created most strains used in the work. Hiro Okano performed enzymatic assays.

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ABSTRACT OF THE DISSERTATION

The Structure of Fitness Landscapes in Antibiotic Resistant Bacteria: Molecular Origins and Evolutionary Consequences

by

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Doctor of Philosophy in Physics

University of California, San Diego, 2013

Professor Terence Hwa, Chair

Antibiotic use is so ingrained in modern healthcare and agriculture that it can be difficult to imagine life in the pre-antibiotic era of the twentieth century. Many privileges we currently take for granted as rights, e.g., modern surgery, would not be possible without these drugs. But the rapid rise of antibiotic resistance may soon thrust the world back into this era. In order to predict (and ultimately prevent) the emergence of antibiotic resistance, it is crucial to establish quantitative, predictive links between the fitness of drug resistant organisms and the molecular mechanisms conferring resistance. Although the resistance

mechanisms are often well characterized in vitro, their contributions to microbial fitness may depend critically on the environment and on the internal state of bacteria, which are often unknown in quantitative terms.

To bridge this gap I investigate *E. coli* strains constitutively expressing resistance to translation-inhibiting antibiotics. The results show that in the presence of drugs, genes providing drug resistance are subject to an innate, positive feedback due to the global effect of drug-inhibited growth on gene expression. This feedback results in complex behaviors for isogenic populations of cells, including an abrupt drop in the growth rate of cultures at a threshold drug concentration. At drug concentrations below this threshold, cells exhibit growth bistability—the coexistence of large populations of non-growing cells among otherwise identical, but growing, cells. This work demonstrates for the first time that many bacteria remain susceptible to an antibiotic even as they carry resistance to it. These behaviors do not appear in strains that lack drug resistance, and a quantitative characterization of drug-drug resistance interactions reveals a whole that is surprisingly richer than its parts.

A mathematical model of bacterial growth based on the innate feedback predicts the onset of bistability and the growth rates of growing sub-populations, without invoking any ad hoc fitting parameters. Furthermore, the model describes a fitness landscape for bacterial drug resistance in different environments, allowing me to characterize the factors that determine the evolvability of resistance. The approach I use can be generalized to study resistance against other classes of antibiotics, besides the translation inhibitors studied here.

Chapter 1

Introduction

1.1 Antibiotics and infection

By 1942, the term “antibiotic” had come into use within the scientific community to describe chemical substances (initially of microbial origin) that inhibit the growth or metabolic activities of bacteria or other microorganisms (Waksman, 1947). With the widespread use of antibiotics in medicine beginning in the 1940s, the U.S. saw a dramatic decline in mortality due to infectious diseases like tuberculosis, pneumonia and syphilis that was unprecedented in the history of the world (Armstrong, Conn, & Pinner, 1999; Cohen, 2000; Cutler & Meara, 2001). The next several decades gave way to a surge in the synthesis and isolation of novel antibiotics, with prescription and (eventual) over-the-counter use of a multitude of new antibiotics following soon thereafter (Conly & Johnston, 2005; WHO, 2012). Scientists labored to understand the way these new compounds worked and established categories for antibiotics based on their activities that are still in use today. Some antibiotics actively kill bacteria when observed *in vitro* and are therefore designated as bactericidal, while others simply halt growth without killing and are designated as bacteriostatic. However, this distinction may be less important *in vivo*, because drugs of both types are effective at eradicating infections. Furthermore,

this distinction is largely concentration-dependent: many bacteriostatic drugs become bactericidal at high enough drug concentrations, and vice versa (Odenholt-Tornqvist, Löwdin, & Cars, 1992; Rahal & Simberkoff, 1979).

A more useful distinction between antibiotics is the mechanism by which they inhibit microbial growth. Most antibiotics fall into one of three broad categories based on their mode of action, cell-wall synthesis inhibitors, DNA/RNA replication inhibitors, or translation inhibitors; translation inhibitors make up a significant fraction of all known antibiotics and have been studied since the 1940s (Bartz, 1948; Jana & Deb, 2006; Madigan, Martinko, & Parker, 2003). These drugs interfere with the process by which genetic information is translated from mRNA into functional proteins, generally by binding to the cell's ribosomes to physically block one or more steps in the process (Wilson, 2009; Yonath, 2005). Although this dissertation focuses in particular on this class of antibiotics because they are well characterized, the overall approach could in principle be applied to other classes as well—as indicated throughout Chapter 3.

1.2 Antibiotic resistance

Due in part to the efficacy of antibiotics, infectious disease is now only the third-leading cause of death in the U.S., and remains second worldwide (Conly & Johnston, 2005; WHO, 2012). However, the widespread use and abuse of these drugs has led to unintended consequences, including the emergence of bacteria that are resistant to treatment by one or more antibiotics. Drug resistance in bacteria is genetically encoded and can be accomplished via drug efflux, enzymatic modification of the drug or its target, changes in permeability, or by various other means (Foster, 1983; Hayes & Wolf, 1990;

Levy, 1992). The short-term consequences of drug resistance have been increased mortality rates (up to 50% higher per infection) and increased costs due to infection (WHO, 2012). The long-term unchecked proliferation of antibiotic resistance could lead to a return to the dark ages of medicine, in which “simple” infections once again become life threatening.

To monitor antibiotic resistance, various standard methods have been adopted over the years that test the susceptibility of bacteria to an antibiotic (Andrews, 2001; Varaldo, 2002). From these tests, researchers typically obtain a single parameter per strain to characterize the degree to which strains are resistant to an antibiotic: the Minimum Inhibitory Concentration (MIC), or one of several related measures (Varaldo, 2002). Unfortunately, the results of these tests can be unrepeatable from one lab to another (Snell & Brown, 2001). Furthermore, *in vitro* susceptibility to an antibiotic does not guarantee *in vivo* susceptibility, and a more quantitatively detailed *in vitro* characterization of antibiotic effectiveness would enhance predictive power of *in vivo* models that take pharmacokinetics into account (Varaldo, 2002). In fact there have been solid efforts to quantitatively model and characterize of the effects of antibiotics on bacterial growth (Regoes et al., 2004). However, these approaches often rely on equations and parameters without clear biophysical interpretations as to their origins. If a model’s variables and parameters do not correspond to some set of physical traits in a bacterium, then the model’s predictive value may be limited when the bacterium’s traits change (as they would during evolution).

1.3 Evolution and fitness landscapes

Ideally, a physically well-motivated model of bacterial growth should provide insight into the evolution of resistance at a quantitative level, answering tough questions like: What will be the new MIC of a mutant bacterium? What is the likelihood that a mutant arising during treatment will outcompete its parental strain? How do the bacterium's physical traits determine the rate at which resistance emerges during treatment? The answers to these questions might depend on many factors; but perhaps the most basic factor is the strain's growth rate in the presence of a given concentration of antibiotic. Then additionally, to characterize evolution, one must know the growth rates of any mutants that might arise, and how these rates depend on the drug concentration in the environment.

In fact, such information would be described by a fitness landscape, a concept popular in the field of evolutionary biology that was first introduced by Sewall Wright (Wright, 1932). Fitness generally denotes an organism's reproductive success—and I take it here to be the organism's proliferation rate (referred to below as the “growth rate”). The fitness of a bacterium will generally depend on the antibiotic concentration in its environment, and on its genetic/phenotypic traits that specify the way in which a drug will interact with the organism. These traits would specify, in the case of translation-inhibiting antibiotics for example, the binding affinity of the drug for the ribosome or the permeability of the bacterium's cell membrane to the antibiotic, etc.

A fitness landscape may be best understood by visualizing two orthogonal groups of axes: along one axis group are all possible biophysical traits of the bacterium (e.g. all possible values of the drug-ribosome binding affinity). The other axis group enumerates

all possible environments (e.g., concentrations of antibiotic) that the bacterium may encounter. Then the fitness at any coordinate in the trait-by-environment hyperplane is specified by the height of a hyper surface extending over the plane; this surface is the fitness landscape. Such a landscape would therefore provide a mathematical mapping from the biophysical details specified by the bacterium's genes to its growth rate. Either a mutation or change in environment for an organism simply corresponds to a change in the location in the landscape.

In this work I characterize the shape, or structure of these landscapes, with particular attention to identifying landscape features that are predicted to arise generically when bacteria exhibit resistance to translation-inhibiting drugs. Although many traits determine the fitness of an antibiotic-resistant strain (leading to an intractably high dimensional fitness landscape (Wright, 1988)), the mathematical model I develop reduces this dimensionality to a space described by only a handful of parameters. These parameters carry straightforward biological significance and permit an intuitive understanding of the axes of the fitness landscapes. The simplicity of this description greatly facilitates the discussion of evolution within these landscapes in Chapter 5. To probe the mathematical description of antibiotic resistance experimentally, I studied the bacterium *E. coli* expressing varying degrees of resistance to the antibiotic chloramphenicol as a model system.

1.4 A paradigm for antibiotic resistance: chloramphenicol and *E. coli*

The bacteriostatic antibiotic chloramphenicol has been around for more than 60 years (Bartz, 1948); so has chloramphenicol resistance (L. L. Cavalli & Maccacaro,

1950). In a 1950 paper on the emergence of *E. coli* resistance to chloramphenicol (called chloromycetin at the time), L.L. Cavalli and G.A. Maccacaro wrote about the differences in the emergence of resistance between drugs perceived to belong to two distinct classes:

The proposal to distinguish two classes of drug resistance—those in which the top level of resistance can be obtained in a single step, and those in which it can be acquired only gradually—has clearly a practical importance; its theoretical meaning has not been entirely elucidated, *since genetical analysis has several limitations in bacteria*. (L. L. Cavalli & Maccacaro, 1950; italics mine)

This quote and the text that follows are relevant to my work for at least two reasons. The first is generic in that a wealth of information on the drug is available because so many talented researchers have had the opportunity to study chloramphenicol and its resistance mechanisms over the years. Of particular importance, and made possible only because “genetical analysis” and molecular biology in *E. coli* has advanced somewhat since 1950, is that many biochemical details about the drug are well characterized at a quantitative level. For example, we know where the drug binds its target on the ribosome to inhibit translation, and with what binding affinity it does so (Harvey & Koch, 1980).

Regarding resistance, researchers have known since the 1960s that a single enzyme is often responsible for chloramphenicol resistance (Chloramphenicol AcetylTransferase, “CAT”), modifying the drug such that it no longer binds its target on the ribosome (Winshell & Shaw, 1969). And by the 1980s William Shaw and others had determined the kinetic mechanisms responsible for the reaction and the kinetic constants that determine the rates at which this enzyme inactivates chloramphenicol (Kleanthous & Shaw, 1984). This detailed level of quantitative information on the drug and the related

resistance mechanism has allowed me to use chloramphenicol resistance as a model, or an exploratory system to test hypotheses and create mathematical models of antibiotic resistant growth in bacteria without having to fit a litany of free parameters, essentially side-stepping one of the most notorious obstacles in quantitative biology today. Using the *in vitro* details described above, and with the collaboration of colleagues, I have been able to

- (i) predict and characterize a novel heterogeneity in the response of antibiotic resistant bacteria to translation-inhibiting antibiotics: growth bistability. This bistability is predicted to arise from an unconventional feedback mechanism identified in this work and is observed to occur for several different drug-drug resistance systems.
- (ii) create a model that quantitatively predicts the growth rates of chloramphenicol-inhibited bacteria that express the CAT resistance enzyme. I show that the same model can be used to predict growth rates of cells resistant to other antibiotics via other mechanisms (i.e. by drug efflux rather than enzymatic modification).
- (iii) predict the increases in fitness associated with increased expression of the CAT enzyme, i.e. construct the fitness landscape

These bullets summarize the integration between experiments and mathematical modeling developed throughout the work.

Now returning to Cavalli and Maccacaro, the second reason their quote is relevant to my work is actually related to (iii) above. Cavalli was interested in the speed at which

“full”, or high-level resistance arises in *E. coli*, and whether this should occur gradually or all at once. To understand these issues, one must face several questions: Which molecular details of resistance determine the rate of adaptation to a drug? What are the likely effects of mutations on these details? Are there any general principles we can glean from these considerations? The theoretical work in Chapter 5 provides a strategy to address these outstanding questions at a quantitative level, and even offers a few answers.

The aims of this work are, in regard to translation-inhibiting antibiotics, (a) to establish the simplest mathematical model of antibiotic-resistant growth consistent with observations, and (b) to convey the evolutionary consequences of the model’s predictions. As I show in Chapter 3, it is only possible to model the effects of translation-inhibition on cell growth because of the extensive work in *E. coli* growth over the past 50 years, allowing for an understanding of the relationships between a cell’s growth rate, its macromolecular composition (RNA and protein per mass, etc.), and its translation rate (Bremer & Dennis, 1987; Schaechter, Maaløe, & Kjeldgaard, 1958; Scott, Gunderson, Mateescu, Zhang, & Hwa, 2010). I also show that to reach my goals (a) and (b), it is critical to understand the activity of a resistance protein within the context of a growing cell: in particular because resistance requires protein expression, but translation inhibition will also affect protein expression (Bollenbach & Kishony, 2011; Davies, Spiegelman, & Yim, 2006; Scott et al., 2010; Scott & Hwa, 2011; C Tan, Marguet, & You, 2009; Washington, 1979; Zaslaver et al., 2009). This two-way interaction creates a feedback in antibiotic resistance that predicts complex behavior, such as the growth-bistability mentioned above in (i). For these reasons I establish the key findings of this work using chloramphenicol, CAT, and *E. coli*, components which are all well-

characterized in isolation but not in interaction together. If we cannot understand the effects of chloramphenicol (Cm) and CAT on *E. coli* growth, we have little chance of understanding much else in antibiotic resistance. But using insights from the Cm-CAT system, I show with experiments and modeling that it is straightforward to generalize the results to other drug/drug-resistance systems and other microorganisms. And while the work focuses on translation-inhibiting antibiotics, the approaches I have developed are generally applicable to understand drug-resistant growth with antibiotics that inhibit growth by other mechanisms; I discuss these issues when appropriate throughout the text.

Chapter 2

The empirical effects of translation-inhibiting drugs on bacterial growth

2.1 Introduction

In this chapter I describe the phenomenology of the bacterial growth response to chloramphenicol and the translation-inhibitor tetracycline in the presence of the expression of proteins conferring resistance to the drugs.

2.2 Growth-mediated feedback in antibiotic resistance

The state of cell growth and gene expression are intimately coupled in exponentially growing bacteria (Bollenbach & Kishony, 2011; Davies et al., 2006; Scott et al., 2010; Scott & Hwa, 2011; C Tan et al., 2009; Washington, 1979; Zaslaver et al., 2009). For example, translation-inhibiting antibiotics have been shown to reduce the expression of even unregulated (or “constitutively expressed”) genes due to growth-mediated global effects (Bollenbach & Kishony, 2011; Scott et al., 2010). If one of these gene products provides some degree of antibiotic resistance, as in the case of drug markers or multidrug efflux pumps (Harbottle, Thakur, Zhao, & White, 2006; X.-Z. Li &

Nikaido, 2009), then a feedback loop is realized in which changes in growth and in antibiotic resistance expression would amplify one another (Figure 2.1). Importantly, this positive feedback may be ubiquitous in nature because many drug efflux pumps are expressed constitutively in the wild (Foster, 1983; Harbottle et al., 2006; Piddock, 2006). Systems with positive feedback often exhibit abrupt responses, together with population level heterogeneity for genetically identical cells (Smits, Kuipers, & Veening, 2006). In the present context, this leads to the prediction of “growth bistability”, i.e., the coexistence of growing and non-growing populations in a homogeneous environment.

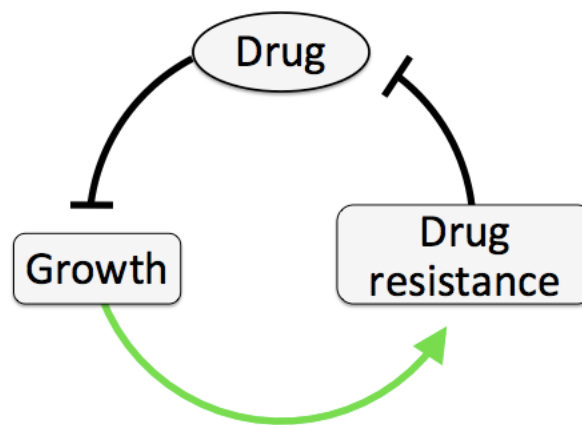


Figure 2.1. Schematic of drug-induced growth bistability.

A proposed positive feedback loop linking antibiotic drug, cell growth, and the expression of drug resistance. The positive link (green arrow) between drug-inhibited growth and gene expression is expected from global growth-mediated effects even for unregulated proteins (Scott et al., 2010).

2.3 Heterogeneity: the difference between antibiotic-sensitive and -resistant growth

To examine the nature of such drug-host interactions and the possible occurrence of growth bistability, I studied the growth of various *Escherichia coli* strains constitutively expressing chloramphenicol acetyltransferase (CAT). CAT is a well-characterized enzyme that modifies and deactivates chloramphenicol (Cm) (Ellis, Bagshaw, & Shaw, 1995); it is often found expressed constitutively in the wild (Shaw, 1983). To assess strains for their sensitivity to Cm, I inoculated cell cultures lacking Cm onto agar plates containing growth medium and various concentrations of Cm, a standard procedure for determining antibiotic sensitivity (Andrews, 2001) (see section 2.7.3). Overnight incubation of CAT-expressing strains on Cm-agar plates indeed revealed signs of population-level heterogeneity. As shown in Figure 2.2 for one such strain, Cat1 (Table C.1), the number of colony-forming units (CFU) decreased gradually on plates with increasing Cm concentrations (Figure 2.2 green bars; Figure 2.3B).

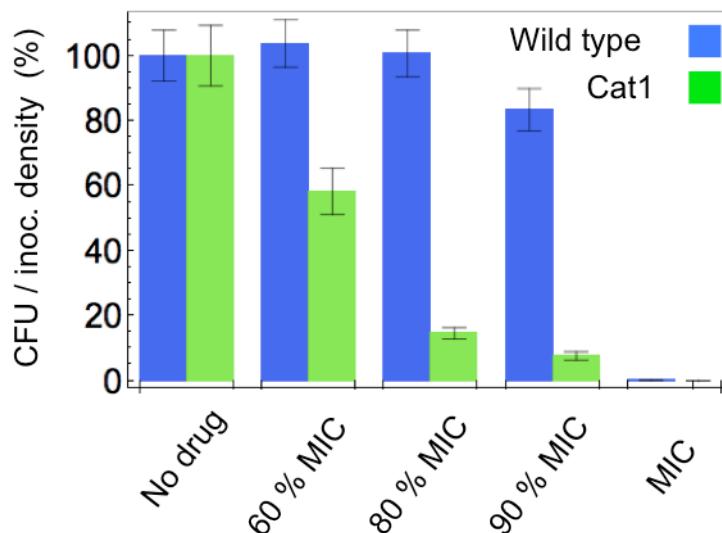


Figure 2.2. Population-level heterogeneity in viability of chloramphenicol-resistant cells.

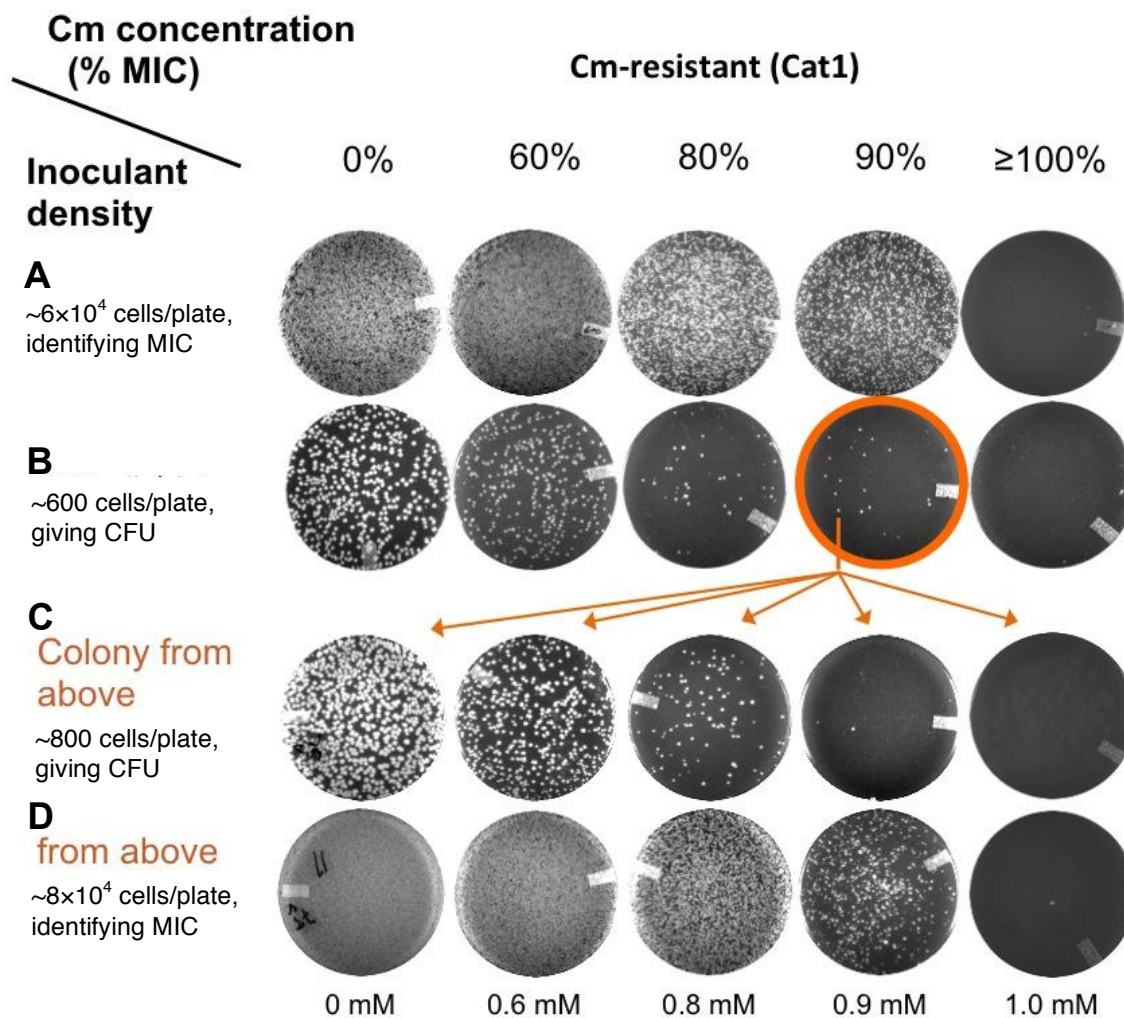
Fraction of viable *E. coli* cells grown on Cm-LB plates for wild type cells (EQ4, blue) and CAT-expressing cells (Cat1, green). The Cm concentration is given as a fraction of the empirically determined MIC_{plate} of the strain, 1.0 mM for Cat1 (Figure 2.3A) and 15 μ M for EQ4 (Figure 2.4A); error bars estimated s.d. from CFU.

Thus, only a fraction of the plated cells formed visible colonies, even at concentrations well below the empirical minimal inhibitory concentration at which colony formation is completely inhibited (MIC_{plate} , Figure 2.3A). I considered that this response might have reflected genetic rather than phenotypic heterogeneity. If genetic variability were responsible for the results in Figure 2.2, then cells that previously grew in the presence of a high Cm concentration would all be expected to grow when exposed again to the same concentration. However, repeating the experiment using a single colony isolated at 90% MIC_{plate} produced qualitatively similar results (with decreased CFU when cells were spread onto plates containing intermediate drug levels, Figure 2.3C-D). In contrast to the heterogeneity exhibited by Cm-resistant strain Cat1, CFU

count of CAT-less wild type cells (strain EQ4) remained high until complete inhibition at MIC_{plate} (Figure 2.2 blue bars, Figure 2.4), indicating that the vast majority of plated cells grew up to the MIC.

Figure 2.3. The apparent MIC and viability of Cat1 cells in chloramphenicol from plate assays.

Typical results for the growth of Cm-resistant (Cat1) cells on LB agar plates containing various concentrations of Cm (shown at the bottom). Cells were diluted from log phase batch cultures lacking Cm, and were spread onto agar at densities indicated on the left of each panel. Plates were incubated overnight for ~18 hours at 37°C; see section 2.7.3 below for details. A, To determine the apparent MIC, referred to as MIC_{plate} and defined as the Cm concentration above which visible colonies formed at a fraction of less than 10^{-4} per inoculum after 18 hours of incubation, the plates are plated at very high densities. The plating results lead to the value of $MIC_{plate} = 1.0$ mM for this strain. Similar results were obtained from replicates. B, 100-fold lower cell density was used to reveal differential fraction of colony formation for the Cat1 strain. The number of colonies formed decreases steadily over sub-MIC Cm concentrations. The fraction of colonies formed per inoculum is plotted in Figure 2.2 (green bars). C-D, The few Cat1 colonies that originally formed on plates with Cm concentration close to 80-90% of MIC_{plate} are not likely mutants. A colony chosen from the indicated plate (orange circle in panel B) was grown in the batch culture and replated on LB agar with the same range of Cm at low or high density (panels C and D respectively). The results obtained are qualitatively similar to those from Cat1 cells that had never been exposed to Cm (compare panels A and B), with CFU decreasing substantially at sub-MIC concentrations.



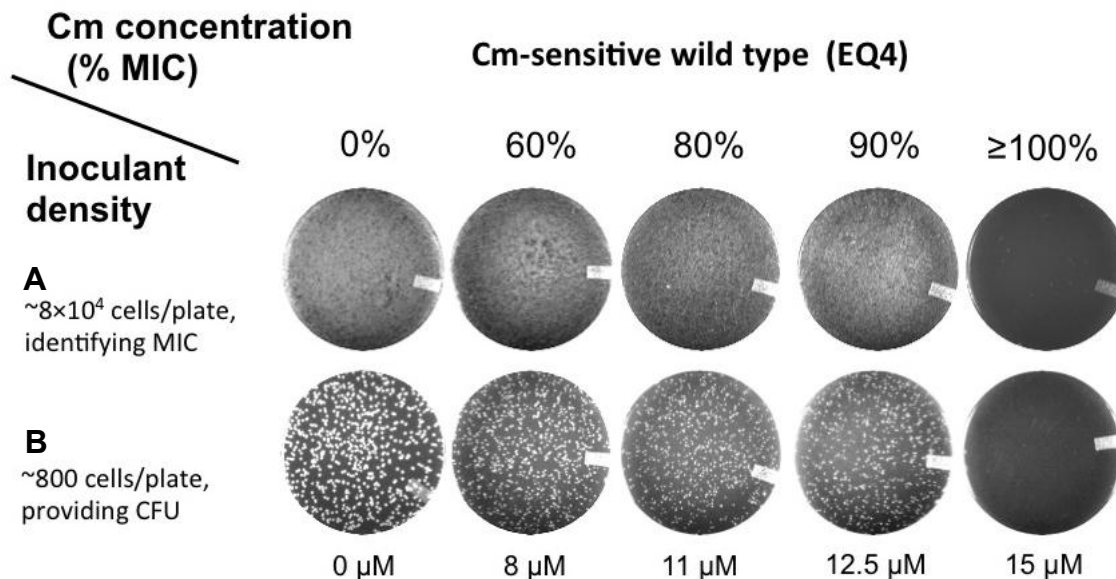


Figure 2.4. The apparent MIC and viability of wild type (EQ4) cells in chloramphenicol from plate assays.

Same as in Figure 2.3 panels A and B but for wild type cells. MIC_{plate} is determined to be $15 \mu M$ from high density plating (panel A). The number of colonies formed on each plate remains nearly constant as the Cm concentration approaches MIC_{plate} (panel B), in contrast to the decreasing CFU count in the Cm-resistant strain *Cat1* at sub-MIC Cm concentrations. The fraction of colonies formed per inoculum is plotted in Figure 2.2 (blue bars).

2.4 Establishing growth bistability with time-lapse microscopy

To verify the coexistence of growing and non-growing cells directly, we employed a microfluidic device in which the growth of individual (immotile) cells could be tracked over extended periods (Groisman et al., 2005) as they grew in the presence of Cm (see section 2.7.4). Immotile CAT-expressing cells (*Cat1m*) grew exponentially in microfluidic chambers before we introduced Cm into the medium. Upon introducing 0.9 mM Cm (90% of MIC_{plate}) into the medium, $\sim 30\%$ of the initial cells continued to grow; non-growing and growing cells were often observed side by side in the same chamber (Figure 2.5). This bimodality persisted for the duration of observations (up to 24 hours), as cells rarely switched between the growing and non-growing states.

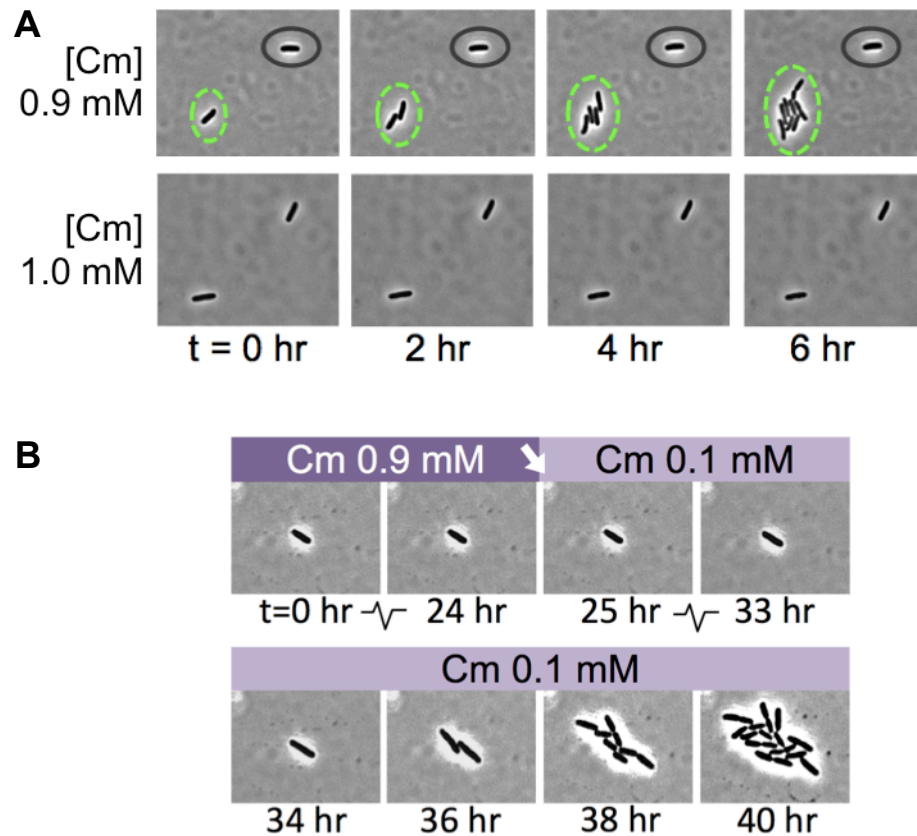


Figure 2.5. Time-lapse microscopy of coexistence: growing and dormant Cm-resistant cells.

A, Upon upshifting from 0 to 0.9 mM Cm in microfluidic chambers (section 2.7.4), genetically identical *Cat1m* cells growing exponentially in glucose minimal medium either continued growing (circled in green) or were growth arrested (circled in black). None of the *Cat1m* cells grew after upshifting to 1.0 mM Cm. **B**, A typical example of cells that remained dormant throughout the 24-hours during which microfluidic chambers contained 0.9 mM Cm; but growth resumed ~10 hours after Cm was reduced to 0.1 mM, which is still above the MIC of wild type cells (see Figure 2.4).

The non-growing cells were viable (or “dormant”), because they resumed growth upon reducing Cm concentration in the chamber from 0.9 mM to 0.1 mM (Figure 2.5), a concentration that completely inhibits the growth of wild type cells (Figure 2.4). Thus the dormant population was stable at 0.9 mM Cm (at least through the 24-hour period tested), but unstable at 0.1 mM Cm.

Repeating this characterization for Cat1m cells at different Cm concentrations revealed that the fraction of cells to continue growing decreased gradually with increasing concentration of the Cm added, (Figure 2.6A green bars), consistent with the Cm-plating results for Cat1 cells (Figure 2.2). Up to 0.9 mM Cm, the growing populations grew exponentially, with the growth rate decreasing only moderately (by up to ~50%) for increasing Cm concentrations (Figure 2.6B). Growing populations disappeared completely for $[Cm] \geq 1.0$ mM, marking an abrupt drop in growth between 0.9 and 1.0 mM Cm (green and black symbols in Figure 2.6B). This behavior contrasts with that observed for wildtype, in which nearly all cells continued growing over the entire range of sub-inhibitory Cm concentrations tested in the microfluidic device (Figure 2.7).

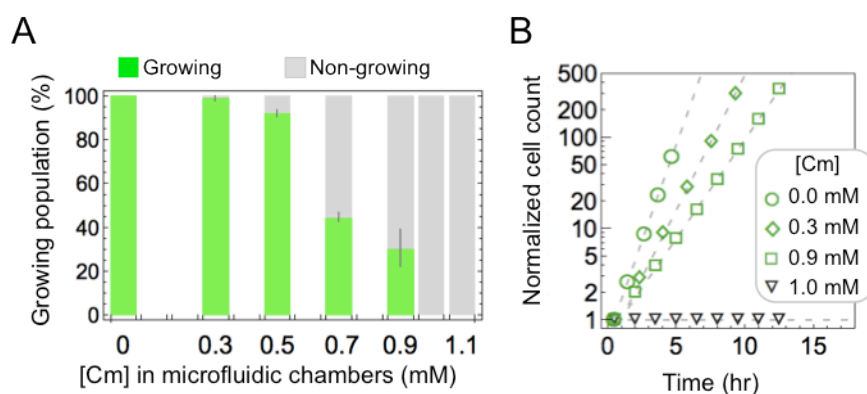


Figure 2.6. Growth of antibiotic-resistant Cat1 populations in microfluidic device.

A, Green bars give the percentage of Cat1m cells to continue growing in microfluidic chambers upon adding indicated concentration of Cm as in panel B. Macroscopic fractions of dormant cells appear between 0.3-0.5 mM (the growth rate of growing cells 0.5 mM was $\sim 0.43 \text{ hr}^{-1}$). Error bars represent s.d. estimates assuming binomial distribution. **B**, The number of Cat1m cells in the growing populations increased exponentially with time. The specific growth rate dropped abruptly from 0.35 hr^{-1} at 0.9 mM Cm to zero at 1.0 mM Cm.

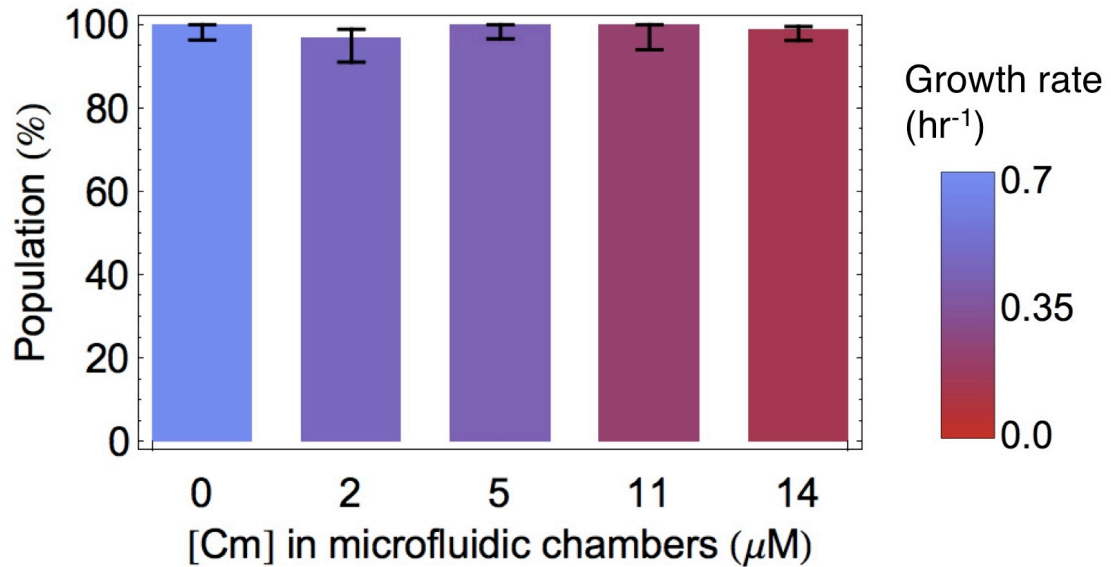


Figure 2.7. *Cm*-sensitive wildtype cells respond uniformly to *Cm* upshift in microfluidic chambers.

Bars give the percentage of wildtype cells (EQ79) to continue growing in microfluidic chambers after upshifting to indicated concentration of *Cm* in glucose minimal medium (contrast with Figure 2.6A). Almost all cells continued growing, with no significant correlation between growth rate and fraction of growing cells ($\rho_{\text{Spearman}} \sim 0.1$). Even at 14 μM *Cm*, with a growth rate less than 0.14 hr^{-1} , ~99% of wildtype cells are growing. In contrast, for the *Cm*-resistant strain, dormant cells appear even as neighboring cells grow rapidly (0.35 hr^{-1} , more than half the rate in absence of *Cm*). Errors are estimated ± 2 s.d. assuming a binomial distribution.

2.5 High-throughput assays to characterize Minimal Dormancy

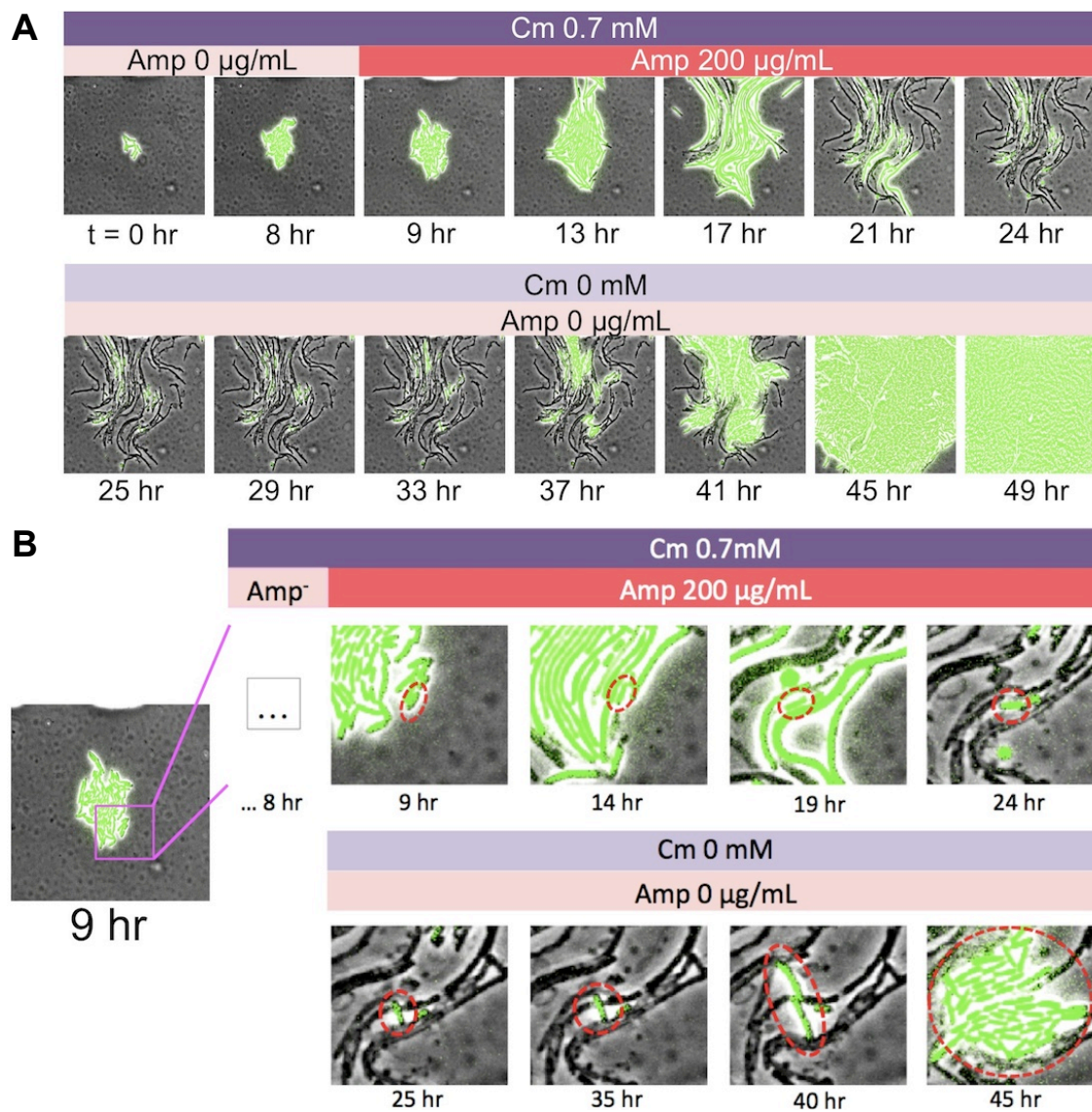
Concentration

Our microfluidic experiments allowed us to directly observe the coexistence of growing and non-growing cells, but usually only a few hundred in any one experiment. To detect dormant Cat1 cells in batch cultures, and to increase the sensitivity range for detecting background dormant cells in wildtype controls, I used a combined *Cm*-ampicillin (Amp) enrichment assay (described below), which takes advantage of the fact

that Amp kills growing cells but not dormant cells (Schlegel & Jannasch, 1967). If a culture contains a heterogeneous mixture of growing and dormant cells and is enriched, only dormant cells survive the enrichment process. Although I ultimately used this technique in batch cultures, here I first present the working principle behind Cm-Amp enrichment in a microfluidic device, directly revealing Amp's bactericidal action against cells that grow in the presence of Cm (Figure 2.8).

Figure 2.8. Time-lapse images of Amp-Cm enrichment in microfluidic chambers.

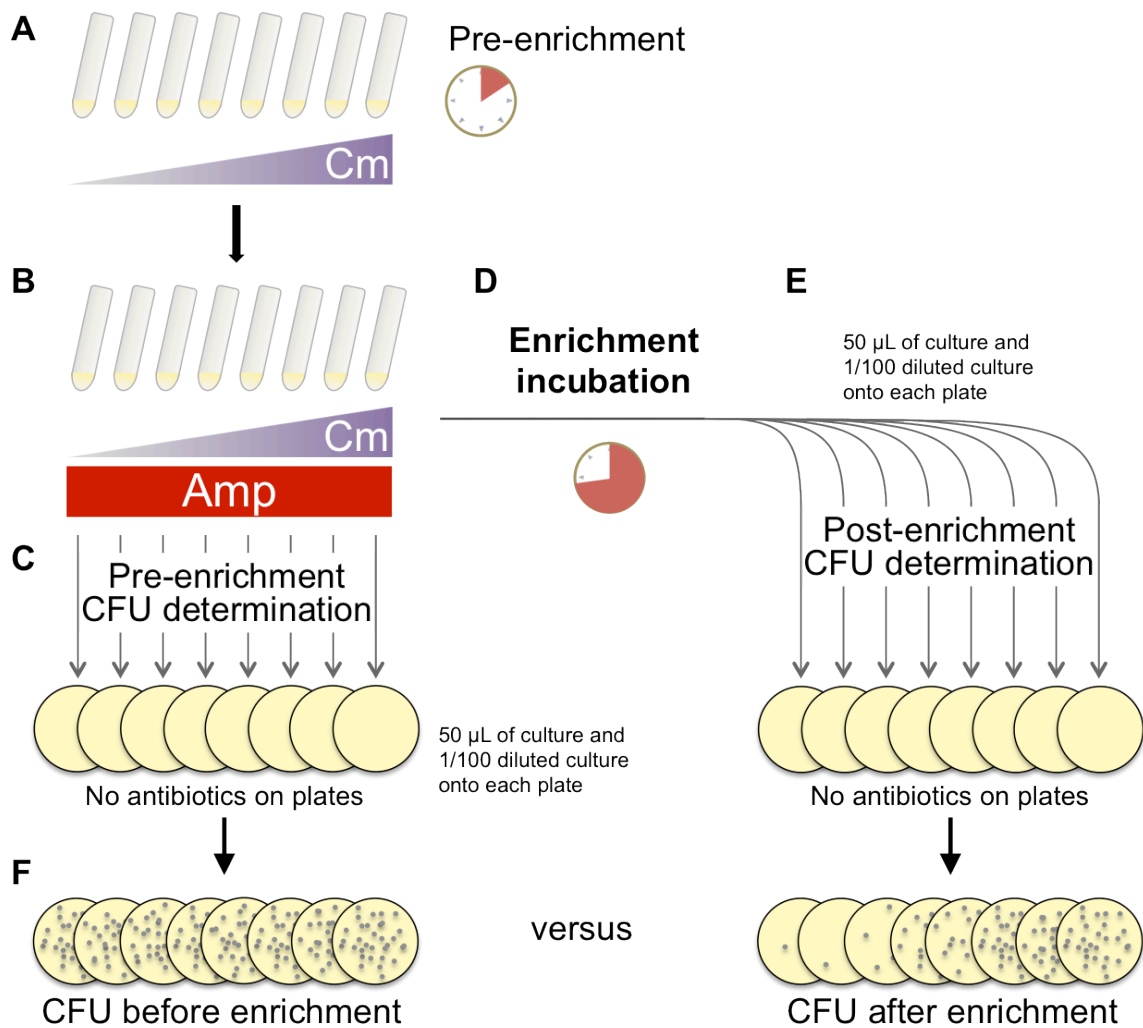
A, This assay was performed in the microfluidic chambers for GFP-expressing *Cat1m* cells (*GCat1m*) growing in minimal medium. We introduced 0.7 mM Cm into the medium at time zero, and added Amp (200 μ g/ml) at $t=9$ hr. By $t=24$ hr, most cells appeared elongated, stopped dividing, and lost fluorescence (characteristic of the loss of cytoplasmic contents accompanying cell lysis and death (Rolinson, Macdonald, & Wilson, 1977). After switching to drug-free medium at $t=25$ hr, cells with fluorescence (those that were not killed by Amp) began growing again, becoming clearly visible after a 12-hr wait ($t=37$ hr). **B**, Detail of (A) shows that only dormant cells survive ampicillin treatment. The history of microcolonies was tracked backwards in time, throughout and prior to enrichment. The red circle shows the history of a representative dormant cell after ampicillin was added at $t=9$ hrs. By visual inspection, we observed that this cell was not growing before and after ampicillin addition at $t=9$ hrs, but began growing after we removed Amp and Cm. Using this technique we found that cells surviving enrichment (and subsequently creating new microcolonies at $t=25$ hr) were always dormant prior to enrichment, with no colonies forming from cells growing during Cm or Amp+Cm treatment.



The batch culture enrichment assay I developed (Figure 2.9) is based on a classic microbiology technique (Schlegel & Jannasch, 1967) and it allowed us to observe the effects of antibiotics on tens of thousands of cells in a single experiment. In the assay, cells were incubated in batch cultures containing various concentrations of Cm along with a bactericidal concentration Amp (100 µg/ml). During this incubation period, cultures become enriched for dormant cells because Amp kills growing cells rapidly or under minimal inhibition but has little effect on non-growing cells (Cozens et al., 1986; Jawetz, Gunnison, Speck, & Coleman, 1951). After incubation, medium from each culture (each containing a unique Cm concentration) was spread onto LB-agar plates lacking antibiotics, to assess the CFU from each culture. Colonies that appeared on these plates arose from cells that were dormant during enrichment (see Figure 2.9 for details).

Figure 2.9. Ampicillin enrichment assay schematic

This assay takes advantage of the fact that ampicillin (Amp) kills growing cells to detect the fraction of dormant cells in a batch culture (Cozens et al., 1986; Griffiths, Miller, Suzuki, Lewontin, & Gelbart, 2000). A, Batch cultures grow in glass tubes containing media with various concentrations of Cm for 1-2 hours to allow cells to acclimate to Cm upshift (from Cm-free preculture). B, Cultures are diluted into identical medium with Cm, together with Amp (100 µg/ml). C, A small volume of culture from each tube is spread onto LB plates lacking antibiotics to determine CFU per culture volume prior to enrichment (the same is done at 100-fold dilution to ensure a countable number of cells). D, Cultures incubate in the amp-enriched media for 6-7 hours. During this incubation period, cultures become enriched for dormant cells as ampicillin kills growing cells rapidly but has little effect on non-growing cells (Cozens et al., 1986). E, Identical volumes of cells from each post-enrichment culture are spread onto LB-agar plates lacking antibiotics. F, Plates incubate overnight at 37°C to reveal colonies formed from cells that remained dormant during enrichment. Comparisons between post- and pre-enrichment CFU (see Figure 2.10) yields the fraction of dormant cells in each culture, plotted in Figure 2.11 for strain Cat1.



Consistent with the microfluidic results (Figure 2.6A), the fraction of dormant cells obtained by the enrichment assay was small ($\leq 10^{-3}$) at 0.3 mM Cm and below (Figure 2.11), comparable to the frequencies characterized for the naturally occurring “persisters” under similar conditions (Balaban, Merrin, Chait, Kowalik, & Leibler, 2004; Lewis, 2007). However, the frequency of dormancy increased substantially at $[Cm] \geq 0.4$ mM (Figure 2.10A, Figure 2.11). I define the ‘minimal dormancy concentration’ (MDC) as the lowest Cm concentration above which significant dormancy appears (indicating at least 1% of the inoculant survives enrichment); $MDC \approx 0.35$ mM for strain Cat1. A conservative cutoff of 1% is used here to avoid false positives due to variability (Luidalepp, Jöers, Kaldalu, & Tenson, 2011) in the naturally appearing persisters (Balaban et al., 2004; Lewis, 2007; Shah et al., 2006) and possible variability in culture density. Thus, growth bistability turns macroscopic fractions of Cm-resistant cells into persisters at Cm concentrations between MDC and MIC. In contrast, enriching Cm-sensitive wild-type cells in Cm reveals that >99% remain sensitive to ampicillin for all sub-MIC Cm concentrations (Figure 2.10B); indeed, wild type cells are only protected from Amp if Cm completely inhibits growth (Cozens et al., 1986; Holm, 1986). Thus, in contrast to what has been theorized previously (for drugs of low membrane permeability (Elf, Nilsson, Tenson, & Ehrenberg, 2006; Fange, Nilsson, Tenson, & Ehrenberg, 2009)), wild-type cells predominantly grew homogeneously in the presence of Cm, and only cells expressing a specific drug resistance enzyme exhibited growth bistability that depended on Cm concentration.

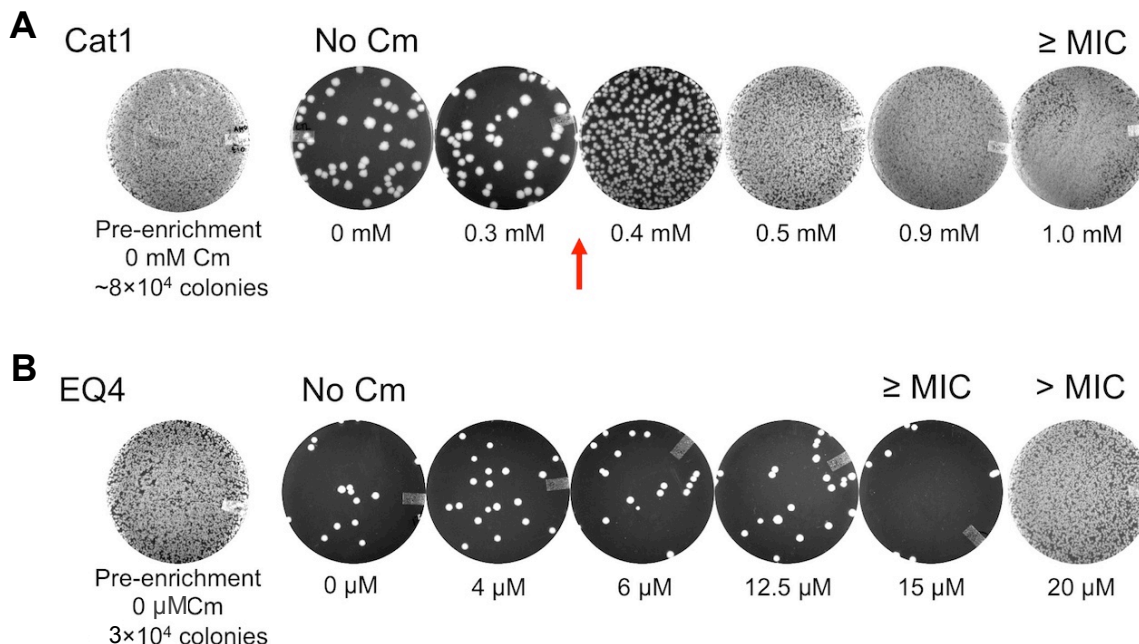


Figure 2.10. The MDC of Cat1 and wild type cells.

Cat1 and wild type (EQ4) cells were treated by the Ampicillin enrichment assay described in Figure 2.9. I measured viable cell densities of $\sim 10^3$ cells per μL culture with dilution plating prior to enrichment (giving $\sim 5\text{--}8 \times 10^4$ colonies/plate, representative results on left). Comparisons between post- and pre-enrichment CFU yields the fraction of dormant cells in each culture. The concentrations of Cm used during enrichment are indicated beneath plates. A, The dormancy frequency is high at and above MIC (≥ 1.0 mM) as expected for Cm-inhibited cells (Holm, 1986). In the absence of Cm, I observed typical “background” dormancy frequencies of $\sim 10^{-3}$ in Amp-cultures as have been reported by others under similar conditions, attributed to the naturally occurring persisters (Balaban et al., 2004; Lewis, 2007; Luidalepp et al., 2011; Shah et al., 2006). Strikingly, colony counts significantly above the background level (> 10 -fold) were obtained from Amp-treated Cat1 cultures, when enriched with concentrations of Cm well below the MIC, e.g., at 0.4 mM Cm. The ‘minimal dormancy concentration’ (MDC), defined as the minimum Cm concentration above which at least 1% of the inoculant survives enrichment, is found to be between 0.3–0.4 mM for this strain (red arrow). A conservative cutoff of 1% is used here to avoid false positives due to variability (Luidalepp et al., 2011) in the naturally appearing persisters (Balaban et al., 2004; Lewis, 2007; Shah et al., 2006) and possible variability in culture density. B, Wild-type cells could not survive ampicillin treatment even with Cm concentrations close to the MIC, e.g., for very slowly growing cultures with $\lambda \sim 0.15 \text{ hr}^{-1}$ at $[\text{Cm}] > 12 \mu\text{M}$ (Figure 2.4). B, Less than 0.1% of wild-type cells formed colonies post-enrichment, for all sub-MIC Cm concentrations used during enrichment (indicated at the bottom), similar to the naturally occurring persisters in Amp enrichment without Cm (Balaban et al., 2004; Lewis, 2007; Luidalepp et al., 2011; Shah et al., 2006). The results suggest that most wild-type cells grew in sub-inhibitory Cm and were therefore killed by Amp. Only at very high Cm concentrations ($\geq 20 \mu\text{M}$) were EQ4 cells significantly protected from the bactericidal effects of Amp during incubation (plate to the far right).

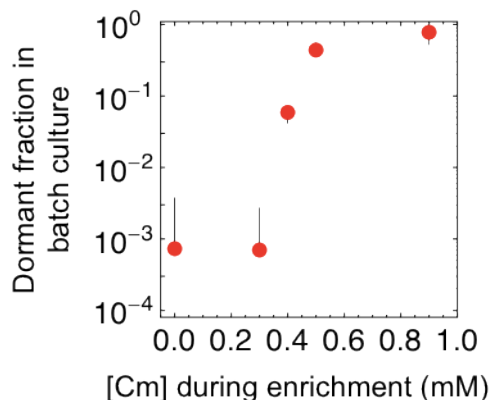


Figure 2.11. Fraction of dormant *Cat1* cells exposed to *Cm* in batch cultures.

Fraction of *Cat1* cells remaining after the Amp-enrichment assay (Figure 2.9), for batch culture growth in minimal medium containing Amp and various concentrations of *Cm*. The results (Figure 2.10) reveal macroscopic fractions of dormant cells for $[Cm] \geq 0.4$ mM, well below the MIC of 1.0 mM. Error bars estimated s.d. from CFU

2.6 The generic appearance of the growth bistability in other strains

We next tested the dependence of growth bistability on the specific action of the drug resistance enzyme. A strain (Ta1) constitutively expressing the tetracycline-efflux pump TetA (Chopra & Roberts, 2001; Schnappinger & Hillen, 1996) was grown in various concentrations of tetracycline (Tc) in microfluidic chambers. As with strain *Cat1* in *Cm*, Ta1 exhibited an abrupt drop in growth rate at MIC (Figure 2.12A) and coexistence of growing and dormant cells for a range of sub-MIC concentrations of Tc (Figure 2.12B). In contrast to Tc-resistant cells, Tc-sensitive cells exhibited smooth reduction in growth rate at sub-MIC Tc concentrations (Figure 2.12C), and no dormant cells were observed even when Tc reduced growth rate by 85%. These results support the hypothesis that growth bistability occurs generically as predicted by growth-mediated feedback (Figure 2.1), independent of the mode of drug resistance.

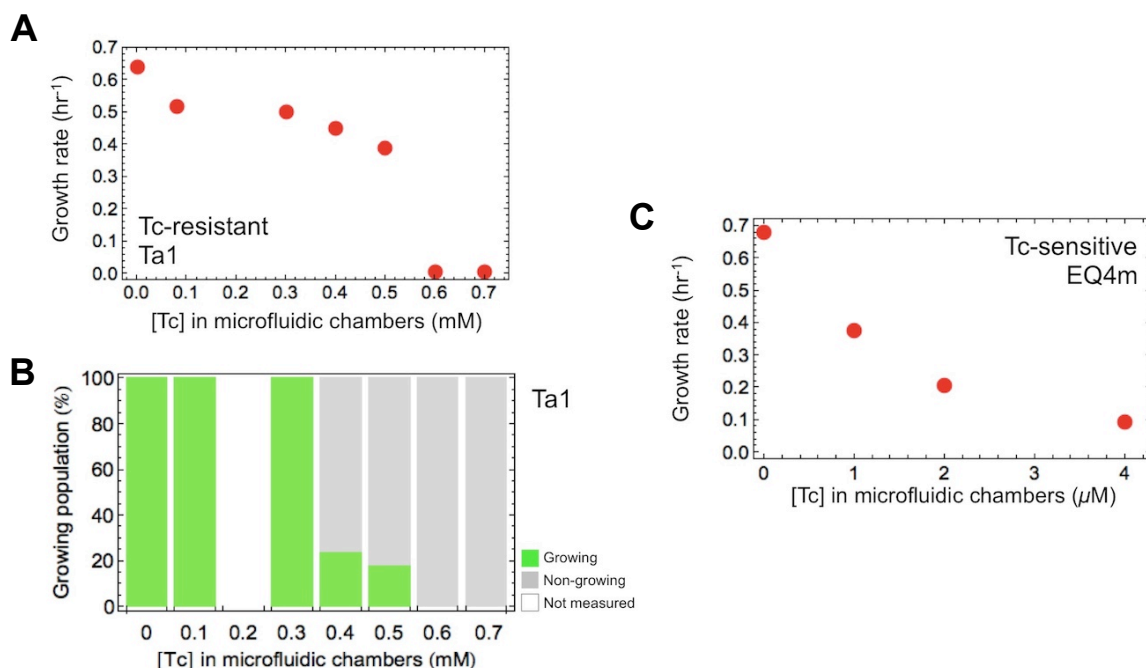


Figure 2.12. Tetracycline-resistant strain *Ta1* showed bimodal behavior when exposed to Tc and wild type did not.

A, Growth rates of tetracycline-resistant strain *Ta1* growing in microfluidic chambers containing minimal medium with indicated concentrations of Tc. Growth rate drops abruptly in the device at 0.6 mM Tc. B, Green bars give the percentage of *Ta1* cells to continue growing in microfluidic chambers after adding indicated concentration of Tc (as for Cm in Figure 2.6). C, Growth rates of wild type (*EQ4m*) in microfluidic device. All wild type cells grew (none were dormant) for all sub-MIC Tc concentrations. Figure 3.8A gives the predicted MIC, MDC, and growth rates for this strain based on growth in batch culture.

2.7 Experimental materials and methods for Chapter 2

2.7.1 Strain construction

All of the strains used in this study are derived from *Escherichia coli* K12 strain MG1655 and described in Table C.1. To create strain EQ4, the region containing *lacI*, *lacZ* and *lacY* genes was deleted from MG1655 using the method of Datsenko and Wanner (Datsenko & Wanner, 2000). DNA oligos used for construct and strain preparation are described in Table C.4. For all experiments, strains stocks were stored at -80 °C. Plates were streaked with stock strains within two weeks of use and stored at 4 °C.

2.7.1.1 Construction of strain *Cat1*

The synthetic and Crp-independent $P_{Llac-O1}$ promoter (-60 to +47, relative to the transcriptional start site) was amplified from pZE12 (Lutz & Bujard, 1997) and substituted for $P_{Ltet-O1}$ in pKDT- $P_{Ltet-O1}$ (Klumpp, Zhang, & Hwa, 2009), yielding pKDT- $P_{Llac-O1}$. The *cat* structural gene, encoding the protein responsible for chloramphenicol resistance, was amplified from pZA31 (Lutz & Bujard, 1997) using forward primer Cat-Kpn-F and reverse Cat-Bam-R. The PCR products were gel purified, digested with *KpnI* and *BamHI*, then ligated into the same sites of pKDT- $P_{Llac-O1}$, yielding pKDT- $P_{Llac-O1}$ -*cat*. Present in this plasmid, the construct $Km^f:rrnBT:P_{Llac-O1}$ -*cat* was amplified using primers Placcat-P1 and Placcat-P2. The Placcat-P1 contains a 51 bp region that is homologous to the intergenic region (272nd to 222nd nucleotides relative to the start codon of *ycaD*) between *ycaC* and *ycaD* while Placcat-P2 contains a 50 bp

region that is reverse complemented to the *ycaC/ycaD* intergenic region located between 116th and 67th nucleotides relative to the start codon of *ycaD*. The PCR products were gel purified and then electroporated into MG1655 cells that carried pKD46 (Datsenko & Wanner, 2000) expressing the λ -Red recombinase. The cells were incubated with shaking at 37 °C for 1 hour and then applied onto LB + Km agar plates. The plates were incubated at 30 °C overnight. The Km^r colonies were verified for the substitution for the *ycaC/ycaD* intergenic region between 222nd and 116th nucleotides relative to the start codon of *ycaD* by colony PCR and subsequently by sequencing. The Km^r:*rrnBT*:P_{Llac-O1}-*cat* construct was moved to background strain EQ4 with P1 phage transduction (Thomason, Costantino, & Court, 2007), and colonies were selected for resistance to Km and Cm on agar plates. The Km^r gene was flipped out using a standard method (Datsenko & Wanner, 2000). The resultant strain is named EQ75, referred to as Cat1 in the text.

2.7.1.2 Construction of strain Ta1

To make the chromosomal *tetA* driven by P_{Llac-O1}, the *tetA* gene was cloned from the transposon Tn10 (also known as *tetA(B)* (Hillen & Berens, 1994)) and subsequently substituted for *cat* in pKDT_P_{Llac-O1}-*cat* (see above), yielding pKDT_P_{Llac-O1}-*tetA*. Similarly as described above, The cassette “Km^r:*rrnBT*:P_{Llac-O1}-*tetA*” was integrated into the *ycaC/ycaD* locus.

2.7.1.3 Chromosomal deletion of *motA* and chromosomal integration of $P_{Ltet-O1}$ -gfp

The *motA* gene deletion in strain JW1879-2 (*E. coli* Genetic Stock Center, Yale Univ.), in which a Km resistance (Km^r) gene is substituted for the *motA* gene, was transferred to EQ4 and Cat1 by P1 transduction (Thomason et al., 2007), and Km^r was then removed as described above to yield EQ4m and Cat1m (Table C.1).

To make the chromosomal GFP driven by $P_{Ltet-O1}$, a *gfpmut3b* structural gene (Levine, Zhang, Kuhlman, & Hwa, 2007) was cloned downstream of $P_{Ltet-O1}$ in pKD13_ $P_{Ltet-O1}$ (Klumpp et al., 2009), yielding pKD13T_ $P_{Ltet-O1}$ -gfp. The construct “ Km^r :rrnBT: $P_{Ltet-O1}$ -gfp” present in this plasmid was integrated into the *intS/yfdG* intergenic region of the MG1655 chromosome as previously described (Kim et al., 2012). The “ Km^r : rrnBT: $P_{Llac-O1}$ -gfp” construct was then P1-transduced into the Cat1m background to create strain GCat1m.

2.7.2 Culture and cell growth

2.7.2.1 Media and chemicals.

Unless noted elsewhere, minimal medium refers to a mixture of glucose 0.4% (w/v), NH_4Cl 20 mM, and “N⁻C” buffer (Csonka, Ikeda, Fletcher, & Kustu, 1994) consisting of 1.0 g of K_2SO_4 , 17.7 g $K_2HPO_4 \cdot 3H_2O$, 4.7 g KH_2PO_4 , 0.1 g $MgSO_4 \cdot 7H_2O$, and 2.0 g NaCl per liter, with 6 mM sodium acetate when indicated. Chloramphenicol (Sigma C0378) stock solutions were either 2 mg/ml or 25 mg/ml Cm in 70% isopropanol stock solution. Tetracycline hydrochloride (Sigma T4062) stock

solutions contained either 0.1 mg/mL Tc • HCl or 25 mg/ml Tc • HCl in deionized H₂O. These stock solutions were stored at -20 °C in the dark and used for preparation of media with various concentrations of antibiotics. Antibiotics were added to media at time of experiment as described below, and for chloramphenicol, stock concentration was chosen such that the volume added would not exceed 1.5% of total media volume.

Prior to use, petri dishes containing ~25 mL LB agar without antibiotics (“LB agar plates”) were stored for up to two weeks after pouring, and kept at 4 °C in a sealed plastic bag to prevent desiccation. LB agar plates with Cm were prepared the day of experiments as follows: after autoclaving freshly mixed LB agar, 100 mL aliquots were poured into 250 mL Erlenmeyer flasks and cooled to approximately 50 °C. A volume of Cm solution was then pipetted from an appropriate stock into the liquid agar (to achieve the desired concentration) and swirled both clockwise and counterclockwise for 10 seconds to mix the agar. I then poured approximately 25 mL medium plus agar into each 100 mm × 15 mm petri dish (Fisherbrand).

2.7.3 Growth of colonies on agar plates

2.7.3.1 Determining CFU on plates with chloramphenicol

For each strain, cells from log phase batch cultures grown in minimal medium lacking Cm were diluted with the same medium. I then used sterile glass beads (Kimble, 4 mm) to spread 50 µL of the diluted culture onto a LB-Cm agar plate to achieve a density of several hundred cells per plate (giving rise to several hundred colonies or

fewer after incubation, depending on the strain's response to the particular Cm concentration used). Plates were incubated overnight (~18 hours) at 37 °C such that colonies formed were easily resolved by the naked eye (see Figure 2.3A-B and Figure 2.4A). I used Bio-Rad Gel Doc XR and Quantity One software to photograph plates and count colonies; in many cases colonies were also counted manually. I calibrated the counting software to agree with manual counts. Plate images were enhanced for brightness and contrast.

2.7.3.2 Determination of MIC_{plate}

Similar to above, cells were diluted from log phase in absence of antibiotics, and 50 μ L of diluted culture were spread onto LB-Cm agar plates to achieve a density of $\sim 5\text{-}8 \times 10^4$ cells per plate before incubation. Plates were incubated overnight (~18 hours) at 37 °C to reveal colony formation. MIC_{plate} is taken as the Cm concentration above which colonies appeared at a frequency of less than $\sim 10^{-4}$ per inoculant; presence or absence of colony growth was readily visually discernable, (see Figure 2.3, Figure 2.4, and Figure 4.5). I determined MIC_{plate} values for each strain after at least two replicate experiments and plate images were enhanced for brightness and contrast. These MIC_{plate} values obtained with LB plates for antibiotic resistant strains were similar to MIC values obtained in batch culture with minimal media as described above. Coincidence between MIC determined in LB and minimal medium has been reported elsewhere (Bollenbach, Quan, Chait, & Kishony, 2009).

2.7.3.3 *Viability after ampicillin enrichment assays*

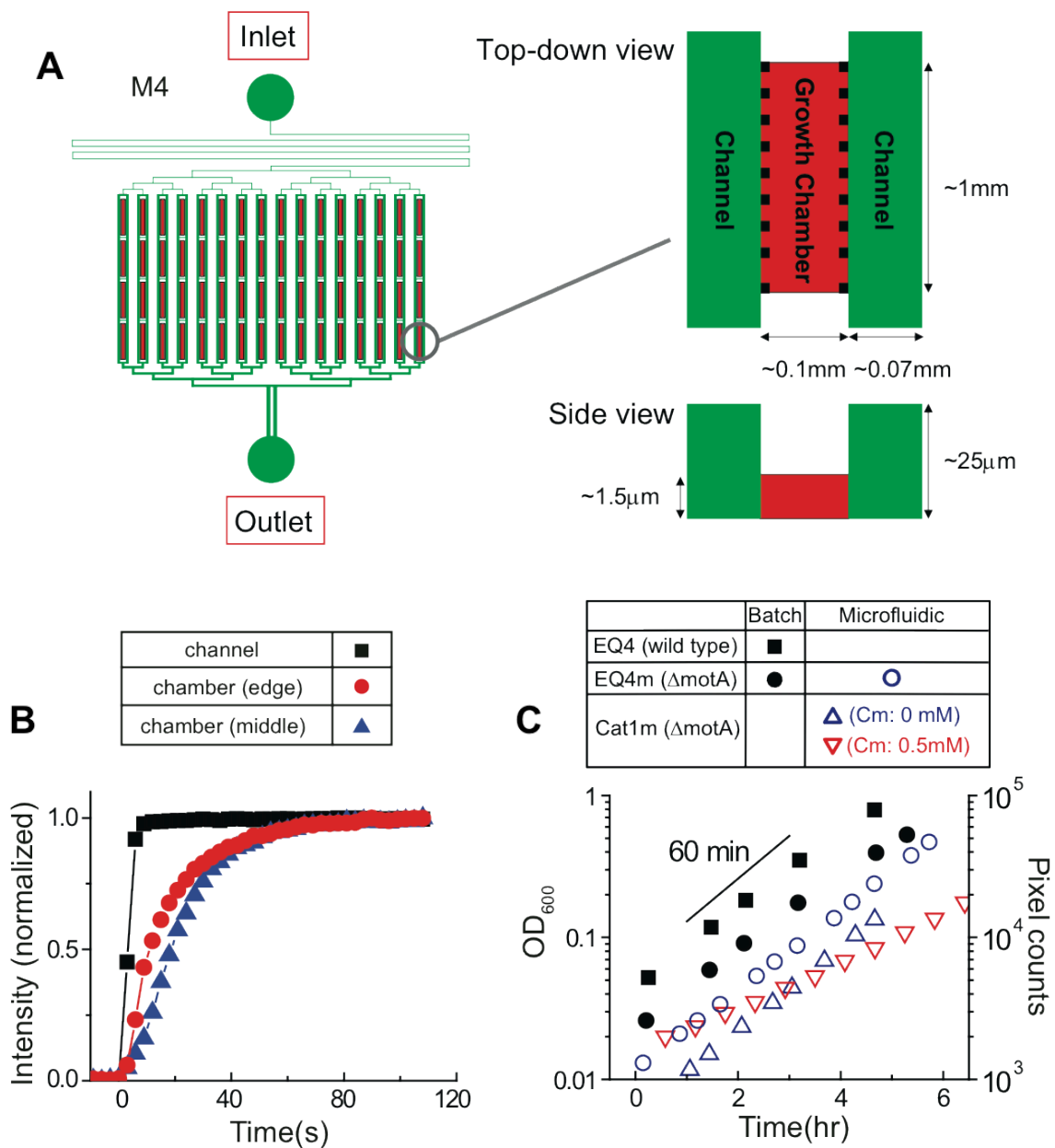
Cells from overnight batch cultures in drug-free minimal media were diluted into the same fresh media with the indicated concentration of “drug” (Cm or Mn as designated in the text) and incubated for 1-2 hours. Cultures were then diluted into identical medium (containing Cm or Mn) with the further addition of Amp (100 µg/ml) to an OD₆₀₀ of $\sim 1\text{-}2 \times 10^{-3}$. At this time, 50 µL aliquots of culture and 100-fold diluted culture were spread onto LB-agar plates lacking any antibiotics and incubated overnight, producing plates containing ~ 500 and $\sim 5 \times 10^4$ colonies each. These plates provide a control to monitor CFU at the start of enrichment and allow us to determine the fraction of cells killed by the enrichment procedure at each drug concentration. After 6-7 hours enrichment in drug and Amp media, 50 µL aliquots of culture and 100-fold diluted culture were again spread onto LB plates without antibiotics for overnight incubation; see Figure 2.9 for illustration. All plates and batch cultures were incubated at 37 °C. Plate images were enhanced for brightness and contrast (see Figure 2.10, Figure 3.9, and Figure 4.6).

2.7.4 Microfluidic device and experiments

The device are described in detail in (Kim et al., 2012).

Figure 2.13 Design and characterization of a microfluidic culturing device for the maintenance of Cm concentration.

We used a microfluidic culturing device to monitor cell growth in a constant environment at the single cell level. The device is similar to a previous design (Groisman et al., 2005), with a few modifications. A Fresh medium flows actively through the outside reservoir (channels, in green) and diffuses into the cell growth chambers (center red in top-down view, $1\text{mm} \times 0.1\text{mm} \times 1.5\mu\text{m}$). The small geometry of microfluidic chambers ensures the fast fluid exchange rate between the reservoir and the chamber. This is shown in panel B, where we abruptly introduced a fluorescent dye (Rhodamine 110, invitrogen) into a channel (black squares) and measured the changes in fluorescence at the edge (red circles) and in the middle (blue triangles) of the chamber. It took ~ 40 sec for fluorescence to reach 90% of its maximum level, suggesting that molecules of similar sizes as the dye are replenished approximately every 40 sec. The fast fluid exchange rate ensures that a constant chemical environment, particularly, the drug concentration, is maintained inside the chamber. This is important because the cells studied here express CAT, which modifies Cm. Thus, the Cm concentration in the growth medium would otherwise decrease over the course of experiment, making it difficult for long-term observation. In order to characterize the growth bistability, we had to track the growth of individual cells for long periods of time (at least 4 cell doublings). To facilitate cell tracking, we used immotile cells (ΔmotA), and immobilized them in each experiment by loosely sandwiching them between the ceiling of the growth chamber and the cover glass, by adjusting the height of the chamber (see below). In panel C, we compared the growth in a microfluidic chamber to that in batch culture. First, wild type (EQ4) cell and ΔmotA (EQ4m) cells grew at similar rates in the batch culture (solid black squares and circles, respectively) and the wild type (solid black square), with ~ 60 min doubling time (line) in minimal medium with glycerol and NH_4^+ . Next, the growth of the ΔmotA strain in the microfluidic chamber was characterized (open red circle) by counting the pixels occupied by growing colonies in phase contrast images; described in detail below). The growth rate is $\sim 10\%$ slower than that in batch culture.



2.7.4.1 Cell growth in microfluidic chambers

All cultures were grown at 37 °C. The growth medium was minimal medium as described above, and was filtered through 0.45 µm filters before use. The cells were first cultured in LB broth in 20 mm test tubes with shaking (250 rpm) in a water bath (New Brunswick Scientific). After 5~6 hrs of growth, they were transferred to the growth medium and grew overnight in the same condition (pre-culture). The pre-culture was inoculated with fewer than 10^5 cells/ml so that cells were in an exponential phase at the time of experiment. The next morning, the pre-culture was diluted to a fresh growth medium containing 0.1% BSA (bovine serum albumin, Sigma; BSA prevents cells from binding to surfaces of microfluidic devices) to an optical density (OD_{600}) of ~0.01 as measured on a Genesys20 spectrophotometer (Thermo-Fisher) with the standard cuvette (16.100-Q-10/Z8.5, Starna Cells Incl; ~200 µL per measurement). To load cells into the microfluidic device, the diluted pre-culture was pressurized to 1 ~ 2 psi at the outlet of the device (Figure 2.13A). When the channel and growth chambers were completely filled with the pre-culture, the culture was removed and a fresh growth medium was introduced from the inlet of the device.

The microfluidic device was fixed onto a motorized microscope stage equipped with autofocus (Proscan II, Prior) in a fluorescent microscope (Nikon TI-U) that were housed in a microscope incubator (InVivo Scientific). When viewed with a charge-coupled device (CCD) camera (Clara, Andor) with a 60x phase-contrast objective, single cells were dispersed far from each other (more than 100 µm away from each other).

Then -0.5 ~ -1.5 psi of vacuum was applied from the outlet to bring down the ceiling of the growth chambers and loosely sandwich the trapped cells (side view). Since the vacuum induces the fresh medium flow in a channel (flow rate of 50~100 $\mu\text{m/s}$), no additional pressure was applied from the inlet.

After ~2 generations of unperturbed growth at 37°C in the growth chambers, the growth medium with various concentrations of chloramphenicol was introduced from the inlet of the device. The 10 ~ 30 positions that contain a single micro-colony in the view ($\sim 100 \mu\text{m} \times \sim 100 \mu\text{m}$) of CCD with a 60x phase-contrast objective were saved in the motorized stage. Phase contrast images of the growing cells for each position were recorded 2 times per doubling. Fluorescence images were taken once per doubling, immediately after phase contrast images for each position with a Xenon excitation lamp (Sutter Inst.). The images were analyzed with a custom-built Matlab program. First, the program identified pixel positions occupied by cells with phase contrast images, obtained a size of a growing colony in time series for each position and calculated the growth rate of the colony. In order to quantify fluorescence levels, fluorescence intensities over the cell-occupying area identified by phase contrast images were averaged.

2.7.4.2 Enriching Cm-resistant cells with ampicillin in microfluidic chambers

First, cells that constitutively express GFP (GCat1m) were transferred from precultures as described above and grew in medium with 0.7 mM of Cm for 8 hours. Initially, 44% of cells grew with the doubling rate of 130 min, which is similar to growth of Cat1m (Figure 2.6B). We added 200 $\mu\text{g/mL}$ of Amp to the medium at $t=9$ hr to kill

growing cells (Figure 2.8). At $t=24$ hr, all growing cells had stopped growing and lost fluorescence. There were several non-growing cells that kept green-fluorescence. At $t=25$ hr, Cm and Amp were removed from the medium. Between $33 \leq t \leq 37$ hrs, the non-growing cells that kept their fluorescence throughout the enrichment resumed growth.

2.8 Acknowledgement

With permission from the coauthors, chapters 2-5 of this dissertation contain work from a manuscript that has been submitted for publication with the working title, “The innate growth bistability of antibiotic resistance,” 2013, Deris, JB; Kim, M; Zhang, Z; Okano, H; Hermesen, R; Groisman, A; Hwa, T. The dissertation author and Minsu Kim are co-first authors in the submitted work.

Chapter 3

A quantitative model of antibiotic-sensitive and -resistant growth

3.1 Introduction

We introduced the idea of “growth-mediated feedback” qualitatively in Chapter 1, anticipating the existence of growth bistability arising from resistance to translation-inhibiting antibiotics. In Chapter 2 I reported the various empirical results in which strains expressing resistance to either chloramphenicol or tetracycline exhibited a bistable growth response to antibiotic exposure, whereas drug-sensitive strains seemed to respond to the same drugs with either uniform growth or stasis. To determine whether the proposed growth-mediated feedback can account quantitatively for the observed growth bistability in the strains, I develop a simple mathematical model to predict the effect of translation-inhibiting drugs on the growth of cells constitutively expressing drug resistance. I present here in detail the mathematical model for antibiotic-sensitive and -resistant growth, and discuss how bistability arises in the model in both a qualitative and quantitative sense.

I set out to model the physical phenomena that I believed to be relevant to

antibiotic-inhibited growth. The components aren't particularly complex mathematically, but I hope they reflect a care for integrating relevant details across disparate levels of physical description. The model is composed of three components, whose details I analyze individually below before integrating them into the context of a single growth model.

3.2 Overview of a minimal model of bacterial growth

We will study translation inhibiting antibiotics and resistance conferred by enzymes that modify the antibiotic (inactivation), or pump the drug out of the cell and away from its target (efflux). I will begin with the simplest equations that realize my basic assumptions and then add complexity to these models when necessary to better describe physiological conditions in experiments; in many cases, I find that the simplest descriptions capture the experimental findings without further elaboration. Whenever possible, I will also provide independent estimates of the parameters used in the model.

In order to compare model predictions to experimental results, I introduce the mathematical model in the context of the experimental model system, Cm-CAT, whose biochemistry is quantitatively characterized (Ellis et al., 1995). While I will often restrict the discussion to the behavior of Cm and CAT to stay grounded in experiments, the model is not inherently limited to describing a single specific antibiotic or resistance enzyme. So when possible, I will apply the general model to the resistance to tetracycline and minocycline conferred by the efflux pump TetA.

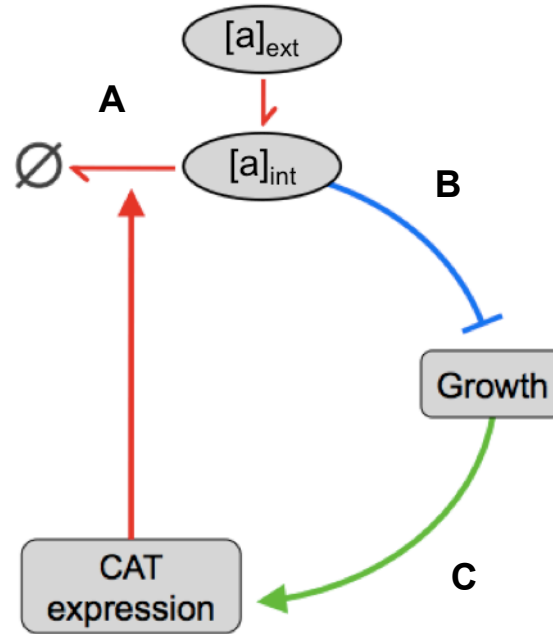


Figure 3.1. Components of interactions defining growth-mediated feedback.

Each link describes a relation substantiated below. (A) The relationship between the internal and external Cm concentration ($[a]_{int}$ and $[a]_{ext}$ respectively) is obtained by balancing the passive influx of Cm into the cell (J_{influx}) with the rate of Cm modification by CAT ($J_{removal}$, Eq. [3.1]), (red arrows). In the presence of an efflux pump or enzyme (like CAT), $[a]_{int}$ can be much lower than $[a]_{ext}$. (B) Translation-inhibiting antibiotics like Cm decrease the steady-state growth rates of cells once they bind the ribosomes inside the cells. (C) The expression levels of constitutively expressed proteins (like CAT) decrease when Cm inhibits growth. Hence, CAT is the critical link in growth-mediated feedback, in which growth rate and intracellular antibiotic concentration influence one another.

I summarize the model here before presenting the full details throughout the chapter. The model contains three components as summarized in Figure 3.1 and the following discussion:

(i) For CAT-expressing cells subject to sub-inhibitory doses of the antibiotic Cm, the steady state relation between the intracellular and extracellular Cm concentration ($[a]_{int}$ and $[a]_{ext}$ respectively) can be obtained by balancing the rates of Cm influx (J_{influx} , diffusion into the cell) and removal ($J_{removal}$, activity of the resistance protein). Any

steady-state growth in the presence of antibiotics must satisfy the simple relation

$$J_{\text{influx}} = J_{\text{removal}} \quad [3.1]$$

by definition, otherwise the intracellular antibiotic concentration would change with time. For the Cm-CAT system, removal is accomplished solely by the activity of the CAT protein, which can be described by Michaelis-Menten kinetics with standard constants V_{max} and K_m (Ellis et al., 1995). Motivations, details, and consequences of this efflux mechanism are treated in section 3.3 below, in which I assume only that removal occurs via standard Michaelis-Menten kinetics, regardless of drug type (e.g. translation inhibitor, DNA replication inhibitor, etc.) or specific mechanism (e.g. efflux, or enzymatic modification of the drug).

(ii) Cells subject to sub-inhibitory doses of antibiotic (Cm) grow exponentially with rate λ (they grow with rate λ_0 in absence of drug). This growth rate should only depend on the intracellular antibiotic concentration ($[a]_{\text{int}}$), which in turn will depend on $[a]_{\text{ext}}$ according to the flux-balance described in (i). (NB, here and throughout this work, the subscript "0" indicates a value in the absence of drug).

(iii) Under translation-limited growth, the expression levels of unregulated genes do not decrease with increasing growth rate as commonly expected from the growth-mediated dilution effect, but are instead observed to decrease linearly with decreasing growth rates λ (Scott et al., 2010). This can be understood as a generic consequence of the up-regulation of genes required for translation (e.g. ribosomal proteins, elongation factors, etc.) upon translational inhibition, at the expense of other genes not directly necessary for translation (described in detail in section 3.5). Therefore, the intracellular concentration of constitutively expressed CAT protein in strain Cat1 is expected to

decrease when Cm inhibits translation, leading to a growth-rate dependent $V_{\max}$. This dependence creates a feedback between growth rate and resistance protein expression. In section 3.5 I present experimental evidence for this growth-rate dependence and discuss its origins in detail, showing that the effect of growth rate on CAT expression can be quantitatively characterized with a simple equation.

Together, the three ingredients above specify a growth-mediated feedback model that makes quantitative predictions of the batch culture growth rates of CAT-expressing cells in Cm-containing media (or more generally, of resistance-expressing cells in the presence of a translation-inhibiting antibiotic). I discuss the model and its predictions in detail below.

Also, I note that bactericidal drugs can elicit complex growth responses such as the “inoculum effect” reported in (Cheemeng Tan et al., 2012), which depends on the cell’s activation of a heat shock response during exposure to aminoglycoside antibiotics. Such responses are not seen with bacteriostatic drugs like Cm and Tc studied here (Kohanski, Dwyer, Hayete, Lawrence, & Collins, 2007; Cheemeng Tan et al., 2012), and are therefore not considered in the model developed below.

Finally, above I introduced $[a]_{\text{int}}$ and $[a]_{\text{ext}}$ as signifying intracellular and extracellular Cm concentration above. However, throughout this work they are intended to represent a generic antibiotic (a), and it will be made clear by context if the variables must be restricted to represent only Cm on a case-by-case basis.

3.3 The relation between intra- and extra- cellular antibiotic concentrations

3.3.1 The relation, in the absence of antibiotic-resistance

Assuming antibiotic enters the cell via passive diffusion, the influx rate is assumed to follow Fick's law

$$J_{\text{influx}} = \kappa \cdot ([a]_{\text{ext}} - [a]_{\text{int}}) \quad [3.2]$$

where κ is the membrane permeability, (units $[\text{time}]^{-1}$) that describes the rate of net influx whenever $[a]_{\text{ext}}$ and $[a]_{\text{int}}$ are not equal. The passive uptake of antibiotics into cells is well-established for Cm (L.M. McMurry, George, & Levy, 1994), Tc (Nikaido & Thanassi, 1993), and many others (Hancock & Bell, 1988). Then in the absence of antibiotic resistance, intracellular antibiotic concentration decreases either by dilution due to growth or by some native low-affinity efflux. I describe the latter as

$$J_{\text{native}} \approx V_{\text{native}}^{\text{max}} \cdot [a]_{\text{int}} / K_{\text{native}}, \quad [3.3]$$

assuming Michaelis kinetics for efflux, with $K_m \gg [a]_{\text{int}}$ for subinhibitory antibiotic concentrations. Note that low-affinity efflux is common in antibiotic-susceptible cells and this endogenous efflux is not associated with clinically relevant antibiotic resistance (L.M. McMurry et al., 1994), which often results from mutations to these endogenous systems. Balancing the influx and efflux in the steady state (and neglecting dilution due

to cell growth¹), I obtain the linear relation

$$[a]_{\text{ext}} = [a]_{\text{int}} \cdot \left(1 + \frac{V_{\text{native}}^{\text{max}}}{\kappa \cdot K_{\text{native}}} \right), \quad [3.4]$$

Since endogenous efflux of antibiotics is weak for cells grown in minimal media (L M McMurry, Aronson, & Levy, 1983), I expect $V_{\text{native}}^{\text{max}} / (\kappa \cdot K_{\text{native}}) < 1$ for Cm (L.M. McMurry et al., 1994) and tetracyclines (L M McMurry et al., 1983; Thanassi, Suh, & Nikaido, 1995) in our experiments.

3.3.2 The relation, in the presence of antibiotic resistance

A key qualitative result in this section is captured by Eq. [3.7], with which I show that the relation between intra- and extracellular antibiotic concentration is marked by two distinct regimes in the presence of an enzyme offering antibiotic resistance. I assume that expression of the gene encoding enzyme E yields a concentration $[E]$ in the steady state, with the effect of reducing the intracellular pool of the active antibiotic. Whether the protein functions as an efflux pump (as in Tc-TetA system) or as enzyme inactivating the drug (as in the Cm-CAT system), I assume the process can be simply described by Michaelis-Menten kinetics, with the rate of antibiotic removal given by

$$J_{\text{removal}} = \frac{V_{\text{max}}}{1 + K_m / [a]_{\text{int}}} \quad [3.5]$$

¹ Typical influx rates are much larger than dilution rate due to growth, at least ~200-fold higher for drugs used in this study (See (Thanassi et al., 1995) and Table C.2)). However, when dilution due to growth is non-negligible, and in the absence of efflux, the dilution may have important consequences for bacterial growth (Elf et al., 2006).

with affinity K_m , and $V_{\max} \equiv k_{\text{cat}} \cdot [E]$. Assuming that the antibiotic enters the cell via passive diffusion (Eq. [3.2]), the balance of influx and removal leads to the following relation between the external and internal drug concentrations:

$$[a]_{\text{ext}} = [a]_{\text{int}} + \frac{V_{\max} / \kappa}{1 + K_m / [a]_{\text{int}}} . \quad [3.6]$$

This relation is sketched in Figure 3.2, and the origin of the approximate threshold-linear form is motivated in its caption.

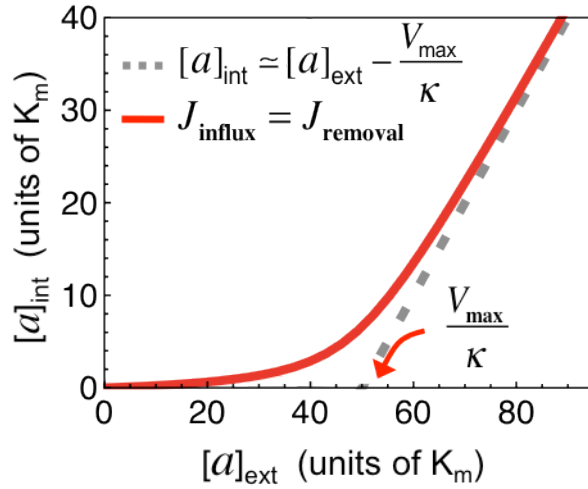


Figure 3.2. The effects of resistance on intracellular antibiotic concentration.

The relationship between the internal and external Cm concentration ($[a]_{\text{int}}$ and $[a]_{\text{ext}}$ respectively), given by the red line, is obtained by balancing the passive influx of Cm into the cell (J_{influx} , Eq. [3.2]) with the rate of Cm modification by CAT (J_{removal} , Eq. [3.5]). This nonlinear relation is characterized by a threshold-linear form, with a “threshold” Cm concentration, $[a]_{\text{ext}}^{\text{threshold}}$ (red arrow), below which $[a]_{\text{int}}$ is kept low as the capacity for clearance by CAT well exceeds the Cm influx; see section 3.3.2. For $[a]_{\text{ext}} \gg [a]_{\text{ext}}^{\text{threshold}}$, CAT is saturated and $J_{\text{influx}} \approx V_{\max}$ (dashed grey line).

Mathematically, the two regimes are simply

$$[a]_{\text{int}} \approx \begin{cases} [a]_{\text{ext}} / (1 + V_{\text{max}} / \kappa K_m) & \text{for } [a]_{\text{ext}} \ll V_{\text{max}} / \kappa \\ [a]_{\text{ext}} - V_{\text{max}} / \kappa & \text{for } [a]_{\text{ext}} \gg V_{\text{max}} / \kappa \end{cases} \quad [3.7]$$

This threshold-linear behavior, when combined with the growth-mediated feedback as described below, provides the mechanism for bistability in antibiotic resistance. An important dimensionless parameter that arises whenever passive influx is balanced by Michaelis-Menten efflux kinetics is $V_{\text{max}} / (\kappa K_m)$, a term that was previously found useful in describing antibiotic resistance (Nikaido & Normark, 1987), albeit in a different context. Strong effect of drug relief is realized for $V_{\text{max}} / K_m \gg \kappa$, where $[a]_{\text{int}} \ll [a]_{\text{ext}}$ for a broad regime of drug concentrations $[a]_{\text{ext}} \ll V_{\text{max}} / \kappa$.

3.3.3 Understanding drug influx in detail

In the analysis of antibiotic resistance above, I made several simplifying assumptions to describe the passive penetration of antibiotics into cells. Here I describe which assumptions may be relaxed without qualitatively changing central results described above. Doing so enables us to provide an independent estimate of the permeability parameter κ to compare with the value fixed by experimental results (see values in Table C.2). I also demonstrate the applicability of the model to more complicated antibiotic resistance systems, using efflux of tetracyclines via TetA as a second model system.

3.3.3.1 *Effective membrane permeability*

First we must appreciate that permeability (κ) in the sense used above depends on both the specific permeability (π) of antibiotic through the membrane, and the surface area (A) of the cell membrane such that the rate of molecules passing through the membrane is given by

$$j_{\text{influx}} = \pi \cdot A \cdot ([a]_{\text{ext}} - [a]_{\text{int}}) \quad [3.8]$$

with π in units of distance per time (lower-case j denotes flux of molecules rather than flux of concentration described by J in Eq. [3.2]). One recovers $J_{\text{influx}} = \kappa \cdot ([a]_{\text{ext}} - [a]_{\text{int}})$, the rate at which intracellular concentration increases in Eq. [3.2], with the definition

$$\kappa \equiv \pi \cdot \frac{A}{\Omega}, \quad [3.9]$$

taking Ω to be the volume of the cell.

3.3.3.2 *A single effective membrane permeability can describe the combined permeability of both the inner- and outer- membranes*

The simplest application for which the use of Eq. [3.2] would be justified is for antibiotics that enter the cell passively by diffusion. However, reality is more complex. Gram⁻ bacteria possess both outer- and inner-membranes, each with their own characteristic permeabilities that in general depend on the levels of porins and low affinity efflux pumps (X. Z. Li, Livermore, & Nikaido, 1994; Mortimer & Piddock, 1993). Despite these complexities, I show here that in many cases, including for the Cm-CAT system, Eq. [3.2] or [3.8] is a reasonably effective model of drug influx at steady

state. Allowing for separate permeability rates of outer and inner membranes (OM and IM), I rewrite the steady-state ($J_{\text{influx}} = J_{\text{removal}}$) as

$$0 = \kappa_{IM} \cdot ([a]_{\text{per}} - [a]_{\text{int}}) - J_{\text{removal}} , \quad [3.10]$$

where $[a]_{\text{per}}$ is the concentration of antibiotic inside the periplasmic cavity, κ_{IM} is the permeability of the inner membrane, and J_{removal} does not depend directly on $[a]_{\text{per}}$. The latter assumption is in accord with the more basic assumption that the resistance mechanism removes antibiotic directly from the cytoplasm according to Michaelis-Menten kinetics. Then, provided that the resistance enzyme removes antibiotic from the cell completely (e.g. not merely expulsion into the periplasm), I can equate the flux of *number of molecules* across each membrane at steady state as follows,

$$\begin{aligned} j_{\text{influx}} = j_{OM} = j_{IM} \Rightarrow \\ \pi_{OM} \cdot A_{OM} \cdot ([a]_{\text{ext}} - [a]_{\text{per}}) = \pi_{IM} \cdot A_{IM} \cdot ([a]_{\text{per}} - [a]_{\text{int}}) \end{aligned} \quad [3.11]$$

Using $j_{OM} = j_{IM}$, one eliminates $[a]_{\text{per}}$ from the above Eq. [3.10] and defines an effective permeability constant

$$\tilde{\kappa} \equiv \frac{A_{IM}}{\Omega_{\text{cyt}}} \cdot \frac{\pi_{IM}}{1 + \frac{A_{IM}}{A_{OM}} \cdot \frac{\pi_{IM}}{\pi_{OM}}} \quad [3.12]$$

to recover the results of Eqs. [3.30], [4.1], and [4.2], with $\kappa \rightarrow \tilde{\kappa}$.

If the resistance mechanism in consideration expels antibiotic into periplasm, as is expected for TetA-mediated efflux of tetracyclines (Thanassi et al., 1995), one obtains an even simpler expression:

$$\tilde{\kappa} \equiv \kappa_{IM} = \pi_{IM} \cdot \frac{A_{IM}}{\Omega_{\text{cyt}}} , \quad [3.13]$$

a result independent of outer membrane permeability at steady state. These results connect the simple permeability parameter κ to the biophysical properties of the cell and motivate the claim that antibiotic influx can be treated according to Eq. [3.2]. Henceforth, I drop the tilde notation with the understanding that κ is used as a “catch-all” parameter in the above sense.

3.4 Effects of antibiotics on growth rate

3.4.1 Effects of antibiotics on translation rate

Whether classified as bacteriostatic or bactericidal, subinhibitory concentrations of translation-inhibiting antibiotics often allow exponential growth in bacteria, albeit at reduced rates (Comby, Flandrois, Carret, & Pichat, 1989, 1988; Garrett & Miller, 1965; Odenholt-Tornqvist et al., 1992). I concern myself here with this simple exponential growth, and model the effect of the antibiotic as reducing the average translation rate γ , due to the direct binding between the ribosome (Rb) and antibiotic (a) inside a growing cell (Harvey & Koch, 1980). I assume that the concentration of antibiotic-bound ribosomes $[Rb \cdot a]$ reaches equilibrium rapidly, characterized by a dissociation constant, K_D . Then the fraction of unaffected ribosomes is

$$\frac{[Rb]}{[Rb]_{\text{total}}} = \frac{1}{1 + [a]_{\text{int}} / K_D}, \quad [3.14]$$

where $[Rb]$ and $[Rb]_{\text{total}} \equiv [Rb] + [a \cdot Rb]$ are respectively the unaffected and total ribosome concentration, and $[a]_{\text{int}}$ is the internal antibiotic concentration. Here I have described equilibrium antibiotic-ribosome binding without cooperativity for simplicity,

but this form is in fact the correct form for the antibiotics used in this work Cm, Tc, and Mn (Epe & Woolley, 1984; Harvey & Koch, 1980). Assuming that the drug-bound ribosomes do not translate at all, the total translation rate in a cell is given by $\gamma_0 \cdot [Rb] = \gamma \cdot [Rb]_{\text{total}}$, where

$$\gamma \equiv \frac{\gamma_0}{1 + [a]_{\text{int}} / K_D} \quad [3.15]$$

is referred to as the average translation rate, and γ_0 is the translation rate in the absence of drug.

3.4.2 The relation between translation rate and growth rate, and the emergence of the half-inhibition concentration

It has been known for over 50 years that exponentially growing cultures of *E. coli* exhibit an obligatory linear relation (growth law) between the ribosomal content and the growth rate λ , when growth is varied by changing nutrient composition in the medium (Maaløe, 1979). I denote the fraction of a cell's proteome comprised of ribosomal proteins and other translation-related proteins as ϕ_R , allowing this growth law to be rewritten as

$$\lambda = \gamma \cdot (\phi_R - \phi_0) \quad [3.16]$$

in which the proportionality factor γ is established as the translation rate (Scott et al., 2010), and ϕ_0 is an empirically determined constant offset.

To understand how translation inhibition affects growth rate, Eq. [3.16] shows that one must also understand how it affects the proteome fraction ϕ_R . Recently, another

empirical linear relation (or growth law) has been established between ϕ_R and λ if growth is varied by changing the translation rate γ , either by using various translational mutants or by applying sub-lethal doses of translation-inhibiting antibiotics, all under the same saturating nutrient source (Scott et al., 2010),

$$\phi_R = \phi_R^{\max} - \frac{\lambda}{\nu} \quad [3.17]$$

This relation describes a trend of increase in the ribosomal fraction when growth is slowed by translational inhibition; the constant $\phi_R^{\max}$ is a term that represents the maximum proteome fraction allocable to the ribosomal fraction. The proportionality factor ν is related to the rate of nutrient utilization and is fixed for a given saturating nutrient medium (independent of translation rate); it is found to increase for nutrients of increasing quality (Scott et al., 2010). Equation [3.17] shows that the cell shifts its proteome to alleviate translation inhibition by creating more ribosome-related proteins. The allocation of ribosome-related resources is the basis for growth-mediated feedback in antibiotic resistance, and is discussed in detail in section 3.5.

Equations. [3.16] and [3.17] can be used to deduce a simple relation between the growth rate λ and the parameters γ and ν ,

$$\lambda(\gamma, \nu) = (\phi_R^{\max} - \phi_0) \cdot \gamma \frac{\nu}{\gamma + \nu} \quad [3.18]$$

which I write as

$$\lambda = \lambda^{\max} \frac{\nu}{\gamma + \nu} \quad [3.19]$$

and $\lambda^{\max}$ is the maximum growth rate achievable with a theoretically best possible

nutrient. In drug-free medium, the above becomes

$$\lambda_0 = \lambda_0^{\max} \frac{\nu}{\gamma_0 + \nu} \quad [3.20]$$

with $\lambda_0 = \lambda(\gamma_0, \nu)$ as the growth rate of drug-free cells, and $\lambda_0^{\max} \approx \lambda(\gamma_0, \infty) \approx 2.85 \text{ hr}^{-1}$ (Scott et al., 2010). Taking the ratio of Eqs. [3.19] and [3.20], and using Eq. [3.15] for γ/γ_0 yields

$$\lambda = \frac{\lambda_0}{1 + [a]_{\text{int}} / I_{50}} \quad [3.21]$$

where

$$I_{50} = K_D \cdot \lambda_0^{\max} / \lambda_0, \quad [3.22]$$

is the predicted half-inhibition concentration, the internal antibiotic concentration that reduces growth rate by 50%. Reports of *in vitro* measurements of K_D for Cm-ribosome binding in *E. coli* range from 0.6 to 2.7 μM (Contreras & Vázquez, 1977; Harvey & Koch, 1980). For wild-type *E. coli* (EQ4) cells growing in glucose minimal media, $\lambda_0 \approx 0.67 \text{ hr}^{-1}$. Eq. [3.22] thus predicts I_{50} to be of several micromolar ($2.8 \leq [I_{50}] \leq 12 \text{ } \mu\text{M}$) during Cm-inhibited growth, as long as the dominant mode of action for the antibiotic is to inhibit translation via ribosomal binding. Inverting Eq. [3.21] leads to the result that the relative doubling time ($\tau/\tau_0 \equiv \lambda_0/\lambda$) increases linearly with the drug concentration

$$\frac{\tau}{\tau_0} = 1 + \frac{[a]_{\text{int}}}{I_{50}} \quad [3.23]$$

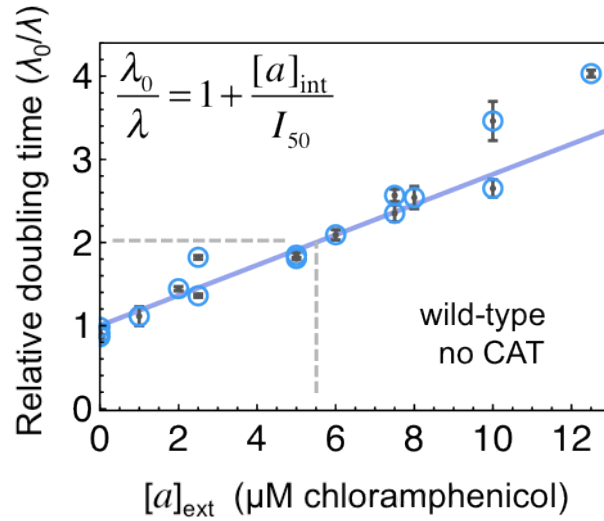


Figure 3.3. The effects of antibiotics on growth in a Cm-sensitive strain.

The doubling time (blue circles) of wild type (EQ4) cells grown in minimal medium with Cm increases linearly with the concentration of Cm in the medium $[a]_{\text{ext}}$ (Eq. [3.24]). I_{50} (dashed vertical line) gives the Cm concentration at which cell growth is reduced by 50%. Here, $[a]_{\text{int}} \approx [a]_{\text{ext}}$ due to the absence of endogenous Cm efflux for wild type cells in minimal media (L.M. McMurry et al., 1994) (see also Eq. [3.4]). Each point represents a single experiment; error bars of the doubling times are standard error of inverse slope in linear regression of $\log(OD_{600})$ versus time.

Growth data in Figure 3.3 verifies the linear relation between doubling time and extracellular Cm concentration for wild type cells, which would be expected provided $[a]_{\text{int}} \propto [a]_{\text{ext}}$ over some broad range of drug concentrations. Equation [3.4] and the ensuing discussion indeed suggest that $[a]_{\text{int}} \approx [a]_{\text{ext}}$ for wildtype strains and Cm. Thus, doubling times of Cm-sensitive strain EQ4 in Figure 3.3 are expected to be described by

$$\frac{\tau}{\tau_0} = 1 + \frac{[a]_{\text{ext}}}{\tilde{I}_{50}} \quad [3.24]$$

with an “effective” half-inhibition concentration, $\tilde{I}_{50} \equiv I_{50} \cdot \left(1 + V_{\text{native}}^{\text{max}} / \kappa \cdot K_{\text{native}}\right)$ defined according to Eq. [3.4], describing external antibiotic concentration when growth rate is

reduced by half. The data of Figure 3.3 give a value of 5.5 μM Cm for the half-inhibition concentration. This is in the range estimated above for I_{50} , suggesting that native efflux may indeed be negligible for Cm at low concentrations in minimal medium.

3.5 The effect of growth-mediated feedback in cells expressing antibiotic resistance

The relation between the internal and external drug concentration shown in Figure 3.2 (Eq. [3.6]) assumes a constant expression level of the enzyme providing drug resistance, i.e., constant V_{max} , as the external drug concentration is varied. However, sub-inhibitory concentrations of antibiotics generically lead to altered expression of proteins (Bollenbach & Kishony, 2011; Bollenbach et al., 2009; Davies et al., 2006; Goh et al., 2002; Scott et al., 2010; Washington, 1979) unless very specific regulatory mechanisms such as negative feedback control are involved (Klumpp et al., 2009). In this work, I investigate the “constitutive” expression of resistance mechanisms, because

- (i) constitutive, i.e. unregulated, expression is the simplest to model and should be understood anyway before attempting to quantitatively understand more complicated systems.
- (ii) in many instances the expression of drug resistance is known not to involve specific regulatory mechanisms (Harbottle et al., 2006), and
- (iii) *de novo* evolution of novel drug resistance mechanisms is likely to initially proceed without drug-specific regulation.

Furthermore, analysis of this seemingly simple system reveals the origins of a complex

growth response to antibiotic. Later, in section 4.5, I discuss the consequences of adding details to the model that increase complexity.

The empirical relation between constitutive enzyme expression and growth rate is shown for translation-inhibited growth in Figure 3.4, with CAT activity ($V_{\max}$) of cells constitutively expressing CAT (open green circles), and LacZ activity of cells constitutively expressing LacZ (open black symbols).

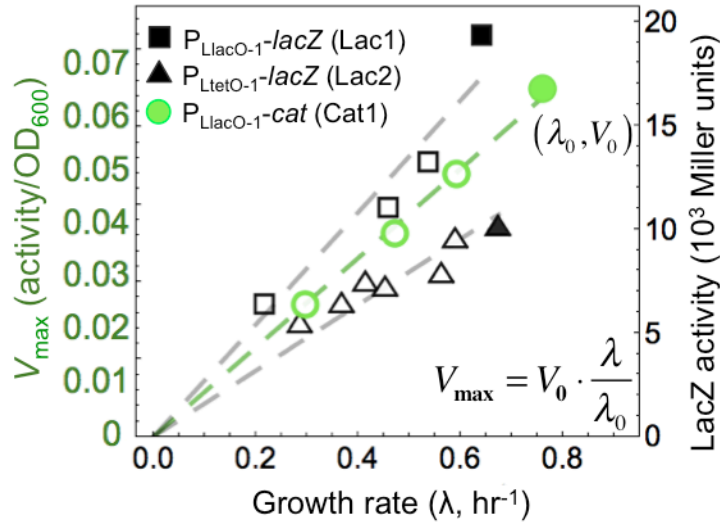


Figure 3.4. The effect of growth on resistance: constitutive enzyme activity decreases with growth rate.

The expression levels of constitutively expressed CAT (green) and LacZ (black) reporters (reported here in units of activity per OD) are proportional to the growth rate for growth with sub-inhibitory doses of Tc and Cm respectively. Here and elsewhere in the text, “expression level” denotes the intra-cellular concentration of a protein. This is reported in units of protein activity per OD: Protein activity is proportional to the protein amount. OD units correspond reliably to cell volume (Donachie & Robinson, 1987; Klumpp et al., 2009; Kubitschek, Baldwin, Schroeter, & Graetzer, 1984).

This result is described in Eq. [3.25], expressed relative to the CAT activity and growth rate in cells not exposed to drugs (denoted by V_0 and λ_0 respectively).

$$V_{\max} = V_0 \cdot \frac{\lambda}{\lambda_0} \quad [3.25]$$

3.5.1 The origins of growth-mediated feedback

The data in Figure 3.4 show that the levels of “unregulated” or “constitutively expressed” genes decrease linearly with the growth rate upon translational inhibition. The rationale for this behavior is that under translational inhibition, the cell allocates growth-limiting resources, particularly the ribosomes, to increase the synthesis of the translational apparatus (signaled by ppGpp (Paul, Ross, Gaal, & Gourse, 2004)). Since most of the ribosomes are engaged in translation according to the bacterial growth laws (Scott et al., 2010), this increase results in decreased synthesis of many other genes, including particularly the unregulated genes. Below I summarize the basic elements of a simple mathematical model that quantitatively predicts this growth-mediated global effect on gene expression, as established by Scott *et al* (Scott et al., 2010).

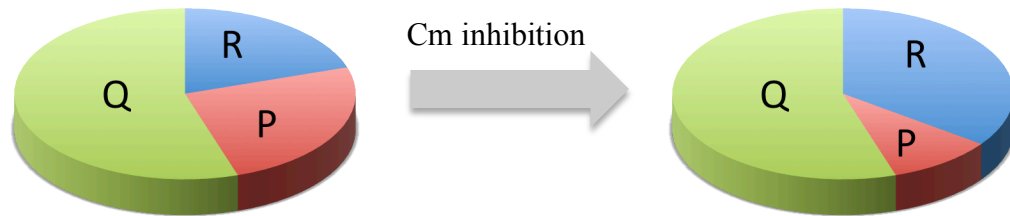


Figure 3.5. Growth-mediated global effects on gene expression.

The model partitions the proteome into 3-sectors (pie chart above), each governed by a distinct growth-rate dependence: (i) The ribosomal sector R , whose mass fraction ϕ_R (blue) decreases as growth is slowed down by nutrient limitation but increases as growth is slowed down by translation. (ii) A growth rate independent sector Q , whose mass fraction ϕ_Q is growth-rate independent (green); and (iii) the remaining sector P whose mass fraction ϕ_P (red) is comprised of what remains after the ribosomal sector changes in response to growth-mediated changes. Because the sector Q mass fraction ϕ_Q remains fixed during growth inhibition, the increase in ϕ_R must come at the expense of ϕ_P . See discussion below.

The model partitions the proteome into 3-sectors (Figure 3.5), each governed by a distinct growth-rate dependence: (i) The ribosomal sector R, whose mass fraction ϕ_R (blue) decreases as growth is slowed down by nutrient limitation but increases as growth is slowed down by inhibition of translation (illustrated by the figure above and to be described in more detail below); (ii) a growth-rate-independent sector Q, whose mass fraction ϕ_Q is growth-rate-independent (green); and (iii) the remaining sector P whose mass fraction ϕ_P (red) is comprised of what remains after the ribosomal sector changes in response to growth-mediated changes, i.e.,

$$\phi_P = 1 - \phi_R - \phi_Q \quad [3.26]$$

Constitutively expressed genes are suggested and shown to belong to the P-sector (Scott et al., 2010). Section 3.4.2 showed that growth rate λ and ϕ_R are related by Eq. [3.17], $\lambda = \nu \cdot (\phi_R^{\max} - \phi_R)$. The value of $\phi_R^{\max}$, found empirically to be ~50% for a spectrum of nutrient sources, is interpreted by Scott *et al* (Scott et al., 2010) to arise from the fixed, growth rate-independent proteome fraction, ϕ_Q

$$\phi_R^{\max} = 1 - \phi_Q \quad [3.27]$$

Substituting from Eqs. [3.17] and [3.27] into [3.26] immediately leads to

$$\phi_P = \lambda / \nu \quad [3.28]$$

for translationally inhibited growth. Eq. [3.28] is the basis of Eq. [3.25] ($V_{\max} = V_0 \cdot \frac{\lambda}{\lambda_0}$),

i.e., the linear relation between constitutive gene expression and the growth rate in Figure 3.4.

This relation (Eq. [3.25]) arises naturally from the repartitioning of a cell's

macromolecular composition due to a change in growth rate. I note here that a growth-rate dependence of macromolecular composition has been observed in a variety of microorganisms (see Fig. S1 in (Scott et al., 2010)), such that some form of growth-mediated feedback is also likely to occur when other organisms are treated with antibiotics. Furthermore, antibiotics that inhibit growth by mechanisms other than translation inhibition will also likely produce some relation between protein expression and growth rate, although it may differ quantitatively from that presented in Eq. [3.25]. For example, subinhibitory concentrations of cephalosporins (cell-wall synthesis inhibitors) have been shown to inhibit production of exoenzymes in *Pseudomonas* (Grimwood, To, Rabin, & Woods, 1989). This is particularly relevant to the discussion here because resistance to cephalosporins is often conferred by the expression of a beta-lactamase exoenzyme (Davies, 1994; Neu, 1985), suggesting that some form of growth-mediated feedback is likely to play a role in this system as well.

3.6 Growth rates and growth bistability in the Cm-CAT system

Combining Eq. [3.25] with Eq. [3.6] gives the full expression relating $[a]_{\text{ext}}$ and $[a]_{\text{int}}$ at different growth rates λ :

$$[a]_{\text{ext}} = [a]_{\text{int}} + \frac{\lambda}{\lambda_0} \cdot \frac{V_0 / \kappa}{1 + K_m / [a]_{\text{int}}} . \quad [3.29]$$

Eliminating λ / λ_0 using Eq. [3.21] gives

$$[a]_{\text{ext}} = [a]_{\text{int}} + \frac{V_0}{\kappa} \cdot \frac{1}{1 + [a]_{\text{int}} / I_{50}} \cdot \frac{1}{1 + K_m / [a]_{\text{int}}} . \quad [3.30]$$

Eq. [3.30] can be inverted to predict the internal drug concentration for every external

drug concentration, given the chemical properties of the drug (K_m , κ , and I_{50}), and the drug resistance expression system that defines V_0 . The value of $[a]_{\text{int}}$ can subsequently be used (via Eq. [3.21]) to predict the relative growth rate (λ / λ_0) for a given external drug concentration.

The model contains three parameters: K_m , I_{50} , and V_0 / κ . The first two are known from literature or can be determined experimentally (Table C.2), while the last one, reflecting the basal CAT activity level (V_0), is construct-specific. In Chapter 4 I will show that the model predicts a precipitous drop of growth rate across a threshold concentration of drug present in the medium, which I identify as the theoretical MIC. Here I report the experimental observation of such a drop for Cat1 cells grown in minimal media batch culture containing various concentrations of Cm (Figure 3.6; see section 3.8.2 below for experimental details). This drop unambiguously defines a batch culture MIC (~1.0mM) that agrees well with the MIC values determined in microfluidics and plate assays of Chapter 2.

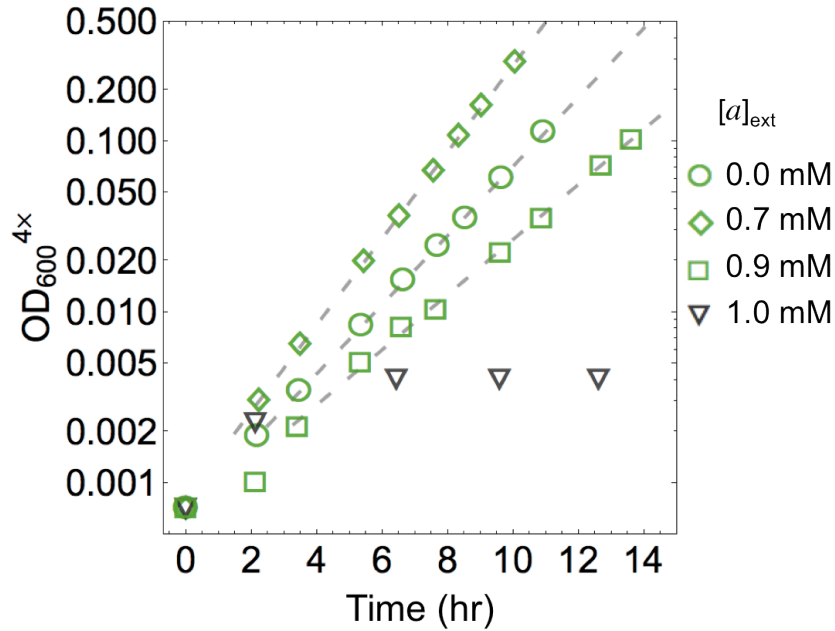


Figure 3.6. Growth curves of *Cat1* cells in batch culture with chloramphenicol.

Representative growth curves showing steady exponential growth in these cultures for up to seven generations, after which the experiments were usually halted. The lines are least squares fit of $\log\text{-OD}$ with time. Inverted triangles show unambiguously that non-growing cultures appear across the MIC. The OD_{600}^{4x} is the optical density as measured using a cuvette with 4 cm light path, allowing four-fold lower cell density to be measured accurately than using standard 1 cm cuvettes. One unit $\text{OD}_{600} = 4 \text{ OD}_{600}^{4x}$.

The model also specifies the way in which the theoretical MIC depends on parameter values. Within the Cm-CAT system, K_m and I_{50} are known as described above, so I seek to fix the lone remaining free parameter for strain *Cat1* using the observed MIC. For the Cm-CAT system, the model predicts $\text{MIC} \approx 0.16 \cdot V_0 / \kappa + 8 \mu\text{M}$ (see Eq. [4.14]). Comparing the empirical MIC value with the predicted dependence of MIC on V_0 / κ fixes this parameter to $\sim 5.8 \text{ mM}$, a value compatible with an independent estimate based on the measured CAT activity V_0 and on indirect estimates of the permeability value κ (Table C.2 and Appendix B). With V_0 / κ fixed, the model predicts Cm-dependent growth rates for this strain without additional parameters (black lines, Figure 3.7).

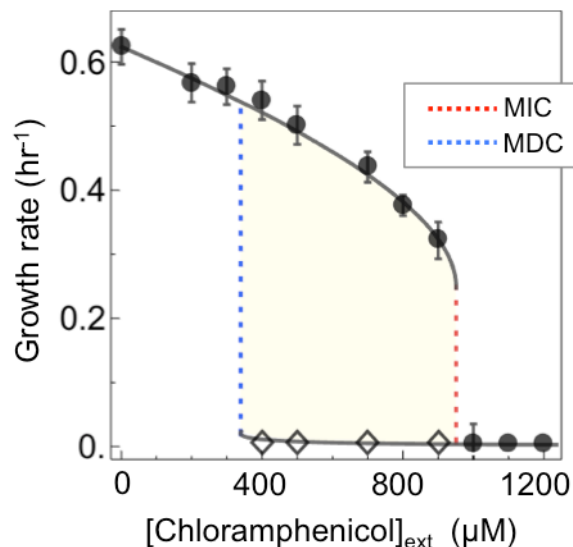


Figure 3.7. Growth rates of Cm-resistant strain Cat1.

Growth rate of Cat1 strain in minimal medium batch culture with varying Cm (filled circles) agrees quantitatively with the prediction of the growth feedback model (line) based on the measured MIC (dashed red line). Error bars s.d.; $n \geq 3$. Dashed blue line is the theoretical MDC. Diamonds indicate drug levels at which enrichment experiments identified significant dormancy (Figure 2.10).

The upper branch of the prediction is in quantitative agreement with the growth rates of Cat1 measured in batch culture (filled circles, Figure 3.7; Figure 3.6).

The model also predicts a lower branch with very low growth rates, and a range of Cm concentrations below MIC where the upper and lower branches coexist (beige band, Figure 3.7). I identify the lower edge of this band as the theoretical MDC because a uniformly growing population is predicted for Cm concentrations below this value. Indeed, the occurrence of dormancy for strain Cat1 as determined in Chapter 2 (open diamonds in Figure 3.7) coincides with the beige band.

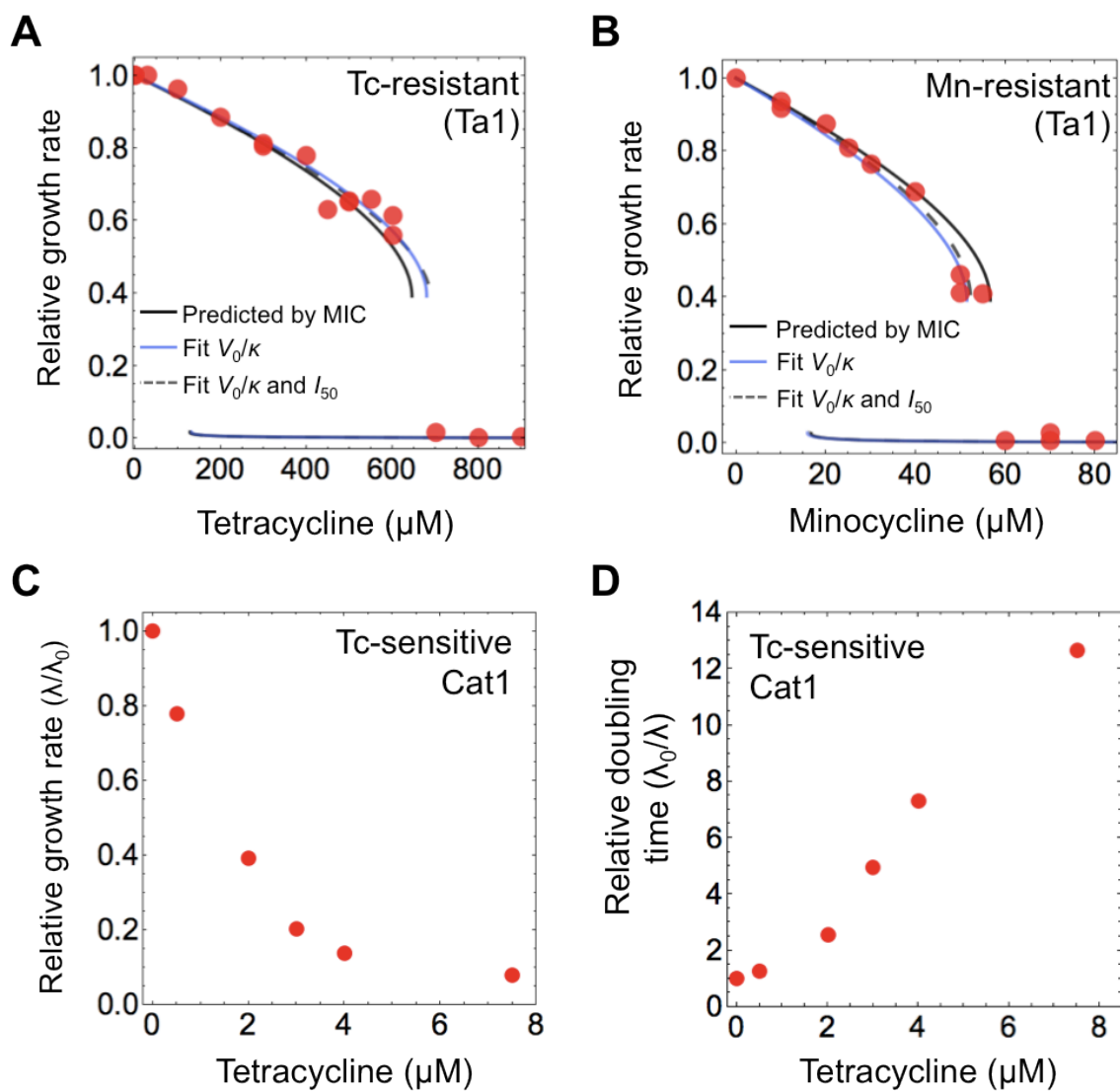
3.7 Predicted growth rates and growth bistability in the efflux of tetracyclines

Our mathematical model based growth-mediated feedback was successful at predicting the growth behavior of strain Cat1 in the presence of Cm. But the model is not intended to describe only the Cm-CAT system; it predicts growth bistability in general for proteins that offer resistance to translation-inhibiting drugs (as outlined above in sections 3.1-3.5). So I tested the dependence of growth bistability on the specific action of the drug resistance enzyme by examining the behavior of other drug resistance systems.

We challenged the tetracycline-resistant strain Ta1 with either Tc or the tetracycline-analog minocycline (Mn) (Chopra & Roberts, 2001) in batch culture and measured culture density over time (as I did for Cat1 and Cm, see section 3.8.2 for details). I plot the results for both drugs in Figure 3.8 along with expected values from the model (we show both the values predicted from MIC and those resulting from fitting the data). The observed growth rates also agreed quantitatively with the upper branch of the respective model predictions made again using MIC to estimate V_0/κ (with other parameters fixed as described in Table C.1). As a control, Figure 3.8 also shows that the growth rates of Tc-sensitive cells do not exhibit an abrupt drop in growth in batch cultures: that is, growth rates decrease smoothly with increasing Tc in the medium. This difference between Tc-resistant and -sensitive cells observed in batch cultures is consistent with the observed growth behaviors in the microfluidic device (Figure 2.12).

Figure 3.8. The MIC and growth rates of Ta1 cells in batch culture with tetracycline or minocycline.

A, The relative decrease in the growth rate of Ta1 cells constitutively expressing TetA in minimal medium with various Tc concentrations (red disks) is well described by the growth feedback model (various lines). The solid black line is drawn with the in vitro value of I_{50}^{Tc} (Table C.5, see also panel **D** below), and V_0/κ_{Tc} as fixed by equation [4.13], with MIC=650 $\pm$ 50 μ M from batch culture growth data (red disks). Alternatively, I fit the model to only the empirical rates of growing cultures (i.e, when $\lambda/\lambda_0 > 0.15$), fitting either V_0/κ_{Tc} alone, or V_0/κ_{Tc} and I_{50}^{Tc} simultaneously as free parameters (blue and dashed gray lines respectively). Fits using growth rates provide parameter values very close to those predicted by MIC, see Table C.5 for parameters and fitting procedures. All growth conditions are described below in section 3.8.2. **B**, Same as (A), except with minocycline instead of tetracycline. Each data point is growth rate from a single experiment, with experiments performed on three separate days. **C**, The relative growth rates of tetracycline-sensitive tetA⁻ strain (Cat1) grown in minimal media with various tetracycline concentrations (red circles). In contrast to Tc-resistant strain Ta1, growth rate does not abruptly drop to near zero across some threshold tetracycline concentration. **D**, Data replotted from (C) shows that relative doubling time increases nearly linearly with drug concentration. Based on in vitro data I predict $I_{50}^{Tc} \approx 4 \mu$ M (Table C.5) such that doubling time should increase by a factor of two at $[Tc]_{ext} \approx 1 \mu$ M (because $[Tc]_{int} \approx 4 \cdot [Tc]_{ext}$, see discussion below Eq. [4.25]). This prediction is consistent with doubling times presented here.



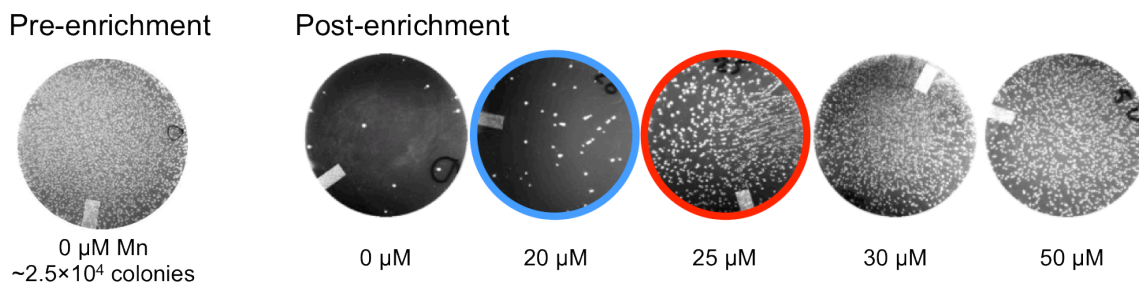


Figure 3.9. Dormancy of Ta1 cells in sub-MIC concentrations of Mn.

Results of batch culture Amp enrichment assay as described in Figure 2.9 and 2.7.3.3, but enriched with minocycline-Amp instead of Cm-Amp. Prior to enrichment incubation, cultures contained roughly 500 cells per μL as verified by control plates (50 μL culture produced $\sim 2.5 \times 10^4$ colonies/plate, see example at left). After enrichment, the same volume of culture contained fewer CFUs in cultures containing 20 μM Mn or less (due to bactericidal activity of Amp). However, above 20 μM many cells remained viable, thus revealing dormant cells below the MIC (identified as 60 μM in batch cultures in Figure 3.8). The MDC for TetA-Mn interaction, defined as the minimum Mn concentration above which at least 1 % of the inoculant survives enrichment, is thus between 20 and 25 μM (between red and blue circles). This is comparable to the model prediction of MDC = 15-30 μM (end of lower branch in Figure 3.8, the value may be as high as 30 μM as described in sections 4.5.3 and 4.5.4).

As another consistency check with the hypothesis that growth-mediated feedback determines cells' growth response to tetracyclines, I confirmed that strain Ta1 exhibited growth bistability during Amp-Mn enrichment experiments (see Figure 3.9 for results and section 2.7.3.3 for experimental details). In fact, microfluidic growth and Amp enrichment experiments with Tc (Figure 2.12) and Mn (Figure 3.9) respectively revealed dormancy within the theoretical coexistence region for strain Ta1 (lower branches in Figure 3.8). I discuss growth and dormancy in tetracycline resistance in detail in section 4.5.

3.8 Experimental materials and methods for Chapter 3

3.8.1 Strain construction

All of the strains used in this study are derived from *Escherichia coli* K12 strain MG1655 and described in Table C.1. Construction of strain Lac2 (also designated as EQ37, in which *lacI* and *lacY* were deleted and *lacZ* is driven by $P_{Ltet-O1}$) is described in Scott *et al* (Scott et al., 2010). Strain Cat1 construction is described in a previous chapter (section 2.7.1). DNA oligos used for construct and strain preparation are described in Table C.4. For all experiments, strains stocks were stored at -80 °C. Plates were streaked with stock strains within two weeks of use and stored at 4 °C.

3.8.1.1 Construction of a new chromosomal *lacZ* reporter strain

The $P_{Llac-O1}$ promoter (described in the preceding chapter) was substituted for $P_{Ltet-O1}$ in pKD13_ $P_{Ltet-O1}$ (Scott et al., 2010), yielding pKD13_ $P_{Llac-O1}$. Using the method of Datsenko and Wanner (Datsenko & Wanner, 2000), the promoter plus the upstream Km^r gene ($Km^r:P_{Llac-O1}$) in pKD13- $P_{Llac-O1}$ was integrated into the chromosome to replace the *lacI* gene and the native *lac* promoter (including the 5' UTR of *lacZ*) of MG1655 deleted for *lacY* (Klumpp et al., 2009). The resultant strain, in which *lacZ* is driven by $P_{Llac-O1}$ and both *lacI* and *lacY* are deleted, is named as EQ5 (also designated as Lac1 in this study).

3.8.2 Culture and cell growth

3.8.2.1 Media and chemicals.

Unless noted elsewhere, minimal medium refers to a mixture of glucose 0.4% (w/v), NH_4Cl 20 mM, and “N⁻C” buffer (Csonka et al., 1994) consisting of 1.0 g of K_2SO_4 , 17.7 g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 4.7 g KH_2PO_4 , 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 2.0 g NaCl per liter, with 6 mM sodium acetate when indicated. Chloramphenicol (Sigma C0378) stock solutions were either 2 mg/ml or 25 mg/ml Cm in 70% isopropanol stock solution. Tetracycline hydrochloride (Sigma T4062) stock solutions contained either 0.1 mg/mL Tc·HCl or 25 mg/ml Tc·HCl in deionized H_2O ; minocycline hydrochloride (Sigma M9511) stock solution contained 10 mM Mn·HCl. These stock solutions were stored at -20 °C in the dark and used for preparation of media with various concentrations of antibiotics. Antibiotics were added to media at time of experiment as described below, and for chloramphenicol, stock concentration was chosen such that the volume added would not exceed 1.5% of total media volume.

3.8.2.2 Batch culture growth

All batch cultures grew at 37 °C in a water bath shaker at 250 rpm (New Brunswick Scientific G76D) with a covered basin to protect photosensitive chemicals (e.g. tetracycline) from degradation and to prevent heat bath from evaporating. Culture growth and measurements performed on separate days began with unique seed cultures each day. Each 5 mL seed culture grew to saturation in LB broth from a single colony on an LB plate. Seed cultures were diluted into 5 mL precultures containing minimal media

and grown overnight without antibiotic. Except as noted below, experimental cultures were diluted from overnight precultures into 5 mL minimal media supplemented with appropriate antibiotics in 20 mm diameter glass tubes. Experimental cultures were inoculated to initial optical density of $OD_{600} \sim 0.01$, as measured by a Thermo Scientific Genesys 20 spectrophotometer, using a Starna Cells quartz cuvette with a 10 mm light path. At intervals ranging from 40 minutes to two hours, I took 250 μ L samples from growing cultures to measure OD_{600} . For growth in tetracycline or minocycline, to control for thermolability or photosensitivity (Loftin, Adams, Meyer, & Surampalli, 2008; Stoichev, Baptista, Basto, Vasconcelos, & Vasconcelos, 2011), I diluted growing cultures 10-20 fold into fresh identical media to verify that culture age did not affect growth rate over the course of the experiments.

3.8.2.3 Batch culture growth with strains expressing CAT in chloramphenicol

We followed the same procedure as described above, except I began experiments with ~60-fold lower cell densities in bulk cultures to avoid significant degradation of Cm by CAT during the course of growth. Briefly, experimental cultures were diluted from overnight precultures into a larger volume of 10 mL minimal media supplemented with appropriate Cm and acetate in larger 25 mm diameter glass tubes. From the larger experimental culture volume we pipetted 1 mL samples into a Starna Cells quartz cuvette with a 40 mm light path to record optical density (OD_{600}^{4x}). Use of the cuvette with longer path length allowed us to observe cultures at four-fold lower densities using the same Genesys spectrophotometer as above. Experimental cultures were inoculated to a

maximum initial density of $OD_{600}^{4x} \sim 0.0007$ determined by the larger cuvette. In this manner, we were able to achieve steady exponential growth observable up to $OD_{600}^{4x} \sim 0.2$ with this cuvette (see green symbols in Figure 3.6).

3.8.2.4 Determination of the growth rate and MIC

Exponential growth curves for all cultures were fit over approximately three or more generations of doubling by linear regression of log-OD values; steady state was not assumed until cultures underwent at least 2 generations of approximately constant exponential growth. When indicated, uncertainty in the calculated growth rate is standard error (SE) of the resultant slope from the simple linear regression. A growth rate of zero indicates cultures failed to grow after at least 12 hours, or stopped growing within several doublings after addition of antibiotic (e.g, see black triangles in Figure 3.6). If results were ambiguous at a particular Cm concentration, for example if a culture appeared not to grow for 6 hours and then exhibited fast growth, the experiment was repeated in full until consistent growth rates were achieved. For chloramphenicol- and tetracycline-resistant strains, I determined MIC by monitoring optical density of batch cultures as described above (see Figure 3.6, Figure 3.7); I determined that cultures contained [antibiotic] $\geq$ MIC if cultures failed to grow, or if growth rate $\lambda \leq 0.1 \text{ hr}^{-1}$. For strains with high levels of antibiotic resistance (most strains), MIC was unambiguous in that growth was undetectable above some threshold concentration (see, e.g, Figure 3.6). I first determined MICs with antibiotic concentrations set at logarithmic intervals before using finer gradations at linear intervals to achieve a determination within $\sim 10\%$. Error

bars in Figure 4.7 are estimated standard uncertainties from experimental procedures, and are equal to or larger than the range of MIC values obtained in $n \geq 2$ independent observations. As the quantitative model is formulated based on growth in batch cultures, I use these MICs determined in batch cultures wherever I provide model predictions or fits. Additionally, the MIC determined on agar plates (called MIC_{plate}, see Figure 2.3, Figure 4.5 and methods in section 2.7.3.2) and in the microfluidic device (Figure 2.6) generally agreed with these determinations.

3.8.3 β -Galactosidase assay

Chloramphenicol-sensitive strains Lac1 (EQ5) and Lac2 (EQ37) express *lacZ* constitutively from the chromosome (Table C.1). We grew these strains in M63 minimal media + 0.4% glucose in presence and absence of Cm inside 20 mm diameter glass tubes, as described above in the section “batch culture growth.” Experimental culture tubes contained up to 10 μ M chloramphenicol to retard growth. During exponential growth (with and without Cm), four to six samples (~ 200 μ L) were collected from each growing culture ($OD_{600} = 0.1$ to ~ 0.8), fast frozen on dry ice and stored at -80°C prior to the assay. β -Galactosidase activity was measured at 37°C by the traditional Miller method (Miller, 1972). The LacZ activity per volume of cell culture (OD) was obtained as the slope of the plot of LacZ activity levels ($\Delta OD_{420}/\text{min}/\text{ml}$) versus OD_{600} to give Miller Units used in Figure 3.4.

3.8.4 CAT assays

The spectrophotometric CAT assay was used to quantify the CAT activities of strain Cat1 when growth was inhibited by tetracycline (Figure 3.4).

3.8.4.1 *Sample collection and cell permeabilization for CAT assays*

We collected samples (at $OD_{600} \sim 0.15 - 0.4$) from cultures growing in minimal medium after at least 3 generations of exponential growth and stored them at -80°C for assays the following day. To thaw samples we added an equal amount of extraction buffer (0.2 M Tris, pH 8.0, 10 mM EDTA). We then added toluene to 1% by volume, vortexed vigorously for 15 seconds and allowed cultures to incubate at room temperature for 15 minutes before performing the assays as described below. To identify the range in the assay over which CAT activity was proportional to CAT protein levels, we diluted samples with various volumes of permeabilized wild type cells (EQ4) and assayed activity. CAT activities reported in this study are within the linear dose-response window as identified above.

3.8.4.2 *Spectrophotometric CAT assays*

We used essentially the method of Shaw (Shaw, 1975). The assay was performed by aspirating 15 μL permeabilized sample into 135 μL reaction buffer inside the cuvette of a Thermo Scientific Genesys 20 spectrophotometer preheated to 37°C . The standard reaction buffer contained 150 μL of 4 mg/ml 5,5'-dithiobis-(2-nitro-benzoic acid) suspended in Tris/HCl, pH 8.0, 60 μL each of 20 mM Acetyl-CoA and 5 mM Cm, and

1.08 mL deionized H₂O. I recorded absorbance A_{412} every 6 seconds using VISIONlite software (Thermo Scientific) between minutes 4-8 of the reaction (absorbance increased linearly during the reaction for at least 10 minutes); reported activity was taken as the slope of the absorbance over time divided by the final OD₆₀₀ of cells in the reaction volume (U/OD₆₀₀).

3.9 Acknowledgement

With permission from the coauthors, chapters 2-5 of this dissertation contain work from a manuscript that has been submitted for publication with the working title, “The innate growth bistability of antibiotic resistance,” 2013, Deris, JB; Kim, M; Zhang, Z; Okano, H; Hermesen, R; Groisman, A; Hwa, T. The dissertation author and Minsu Kim are co-first authors in the submitted work.

Chapter 4

The emergence of the MIC and growth bistability

The growth-mediated feedback model described in Chapter 3 makes quantitative predictions on how the MIC and MDC depend on the basal CAT expression of the strain (V_0 / κ). Below, I analyze this mathematical model to develop these predictions. To test these predictions I performed experiments on several strains that each express a different basal amount of the CAT protein. I discuss the results of these experiments at the end of this chapter.

4.1 Definitions in the model

Since the relative growth rate (λ / λ_0) only depends on $[a]_{\text{int}} / I_{50}$, and I_{50} itself does not depend on the concentrations of the drug or drug resistance enzyme (see Eq. [3.22]), I rewrite Eq. [3.30] in terms of $a_i \equiv [a]_{\text{int}} / I_{50}$ and $a_e \equiv [a]_{\text{ext}} / I_{50}$, with

$$a_e = a_i \cdot \left(1 + \frac{1}{1 + a_i} \cdot \frac{\rho}{1 + \sigma \cdot a_i} \right) \quad [4.1]$$

Here, I have introduced two dimensionless parameters: the 'resistance efficacy'

$$\rho \equiv V_0 / (\kappa \cdot K_m) \quad [4.2]$$

which indicates the efficiency of the drug-resistance enzyme in unit of drug influx, and the ‘saturability’

$$\sigma \equiv I_{50} / K_m \quad [4.3]$$

which characterizes the extent to which the activity of the drug-resistance enzyme is saturated at growth-inhibiting concentrations of the antibiotic. The relative growth rate,

$$\frac{\lambda}{\lambda_0} = \frac{1}{1 + a_i} \quad [4.4]$$

is now completely specified by a_e , ρ , and σ .

For a given strain growing in a medium with a particular antibiotic and expressing some degree of resistance to that antibiotic, in vitro parameters uniquely determine the values of σ and ρ ; see Table C.2 and Table C.5 for biochemical parameters of the specific systems studied in this work (Cm-CAT, Tc-TetA, Mn-TetA). Here I characterize the generic behavior of the system, i.e., the dependence of cell growth on external drug concentration as determined by Eq. [4.1], with σ and ρ as arbitrary parameters.

4.2 Criteria for the coexistence of multiple growth phases

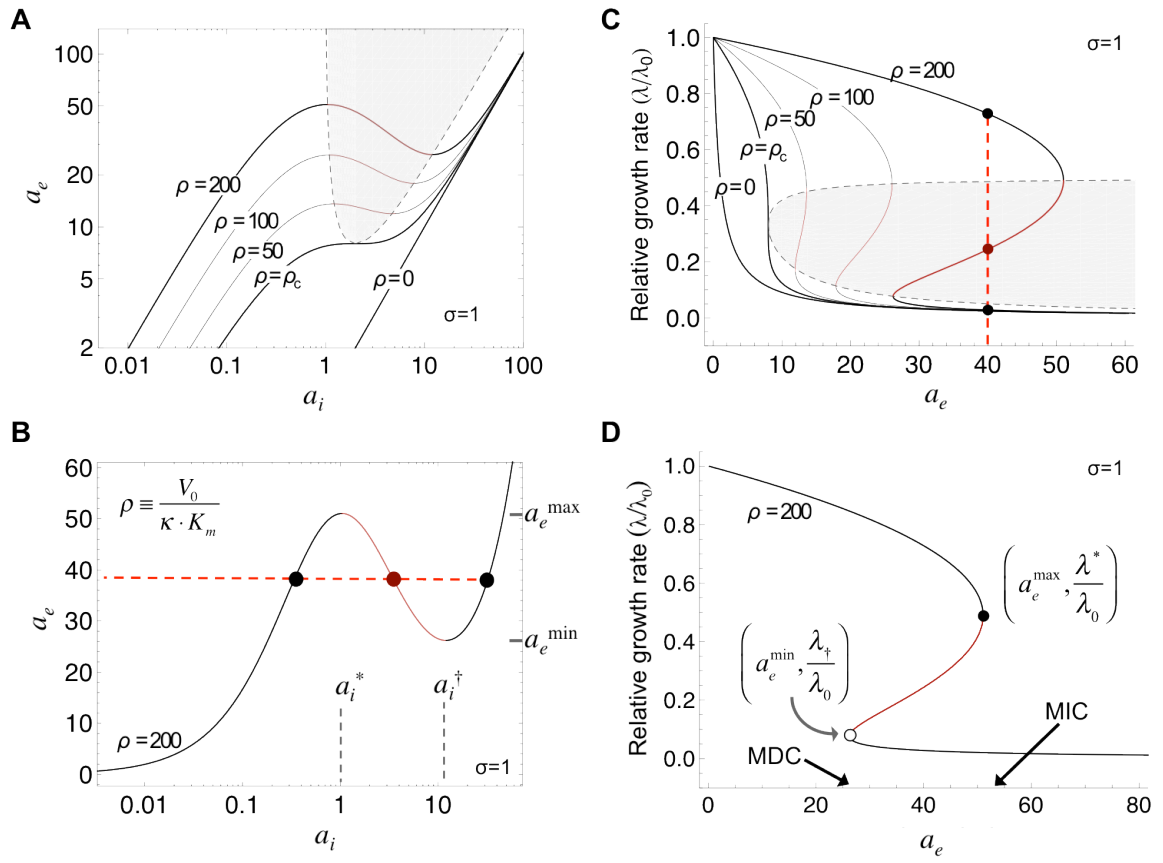
4.2.1 Origin of coexistence

Plotting the relation between a_e and a_i as defined by Eq. [4.1] for a range of ρ values, we see that for sufficiently large ρ , there exists a range of external antibiotic concentration a_e for which multiple a_i values correspond to a single a_e for a given ρ (Figure 4.1A). Since each internal concentration a_i corresponds to a unique growth rate

according to Eq. [4.4], multiple solutions here correspond to the coexistence of multiple growth phases. As indicated in Figure 4.1B for a fixed set of σ and ρ , the appearance of the local extrema $a_e^{\min}$ and $a_e^{\max}$ define the boundaries of external drug concentrations within which multiple growth phases coexist (for a particular strain/condition that corresponds to the chosen values of σ and ρ).

Figure 4.1. Growth bistability predicted by growth-mediated feedback in drug resistance.

Model predictions for the dependence of the growth rate (λ) and the intracellular antibiotic concentration ($[a]_{\text{int}}$) on the external antibiotic concentration ($[a]_{\text{ext}}$) at steady state. The concentrations are plotted in units of I_{50} , i.e., $a_i = [a]_{\text{int}} / I_{50}$ and $a_e = [a]_{\text{ext}} / I_{50}$. There are two dimensionless parameters: $\rho \equiv \frac{V_0}{\kappa \cdot K_m}$ is the resistance efficacy and $\sigma \equiv I_{50} / K_m$ is the saturability (see Eqs. [4.2] and [4.3]). Saturability is of the order 1 for both Cm and Tc studied in this work (Tables C.2, C.5), i.e. $K_m \sim I_{50}$ such that the enzyme activity approaches V_{max} prior to complete growth inhibition. For simplicity, I use $\sigma = 1$ for all plots shown here. A, The theoretical relation between a_i and a_e in the model is shown for a range of resistance efficacies ($0 \leq \rho \leq 200$) to give a sense of scale of this parameter. The family of curves show the region with multiple solutions (i.e., 3 values of a_i for the same a_e) for $\rho > \rho_c$, where $\rho_c = 27$ for $\sigma = 1$ according to Eq. [4.6]. B, The same relation is shown for a strain with high resistance efficacy ($\rho = 200$). The red dashed line shows that a single extracellular concentration may map to three intracellular concentrations in the region between a_e^{min} and a_e^{max} . However, only two are predicted to be stable solutions; intermediate internal antibiotic concentrations (red segments throughout the figure) are kinetically unstable (see stability analysis in the Appendix). The intracellular concentrations corresponding to a_e^{min} and a_e^{max} are a_i^+ and a_i^* , respectively. C, Plots of the growth rate (relative to the drug-free case, λ_0) as a function of a_e for various values of resistance efficacy, ρ . Note that solutions for the growth rate in the grey region are predicted to be kinetically unstable and, therefore, unobservable in experiments (red segments). D, Relative growth rate as a function of external antibiotic concentration for $\rho = 200$. The growth rates at a_e^{min} and a_e^{max} are denoted as λ_+ and λ^* , respectively (empty and filled circles); see Eqs. [4.13] and [4.20]. The quantities a_e^{max} and a_e^{min} are identified as MIC and MDC respectively.



The two local extrema divide the solution of Eq. [4.1] into three branches of internal antibiotic concentration: low, high, and intermediate, for $a_i < a_i^*$, $a_i > a_i^\dagger$, and $a_i^* < a_i < a_i^\dagger$ respectively, as indicated in Figure 4.1B, with the red curve segment indicating the intermediate region. Figure 4.1C–D show the dependence of the growth rate l on a_e , by converting a_i to l using Eq. [4.4]. Comparing Figure 4.1B and Figure 4.1D shows that the low- a_i region corresponds to sub-inhibitory growth, and the high- a_i region corresponds to growth inhibition, though the growth rate in this region is small but finite according to the model. In the section below, I show that $a_e^{\max}$ and $a_e^{\min}$ correspond to the theoretical MIC and MDC respectively (Eqs. [4.13] and [4.20]) for parameter values relevant to this study. The intermediate- a_i region is dynamically unstable (and hence irrelevant) according to deterministic kinetic analysis (see Appendix Eq. [A.1]).

4.2.2 The onset of coexistence at the critical resistance efficacy

Figure 4.1A shows that the local extrema disappear for sufficiently small ρ , i.e. for $\rho < \rho_c$. The bifurcation point at which the extrema just appear, commonly referred to as the “critical point”, can be obtained from the following defining conditions,

$$\frac{\partial a_e(a_{i,c}, \rho_c, \sigma)}{\partial a_i} = \frac{\partial^2 a_e(a_{i,c}, \rho_c, \sigma)}{\partial a_i^2} = 0 \quad [4.5]$$

solving for the critical values ρ_c and $a_{i,c}$ as a function of σ . I find the minimal resistance

efficacy that produces phase coexistence to be

$$\rho_c(\sigma) = \left[\sigma^{1/3} + 1 + \sigma^{-1/3} \right]^3. \quad [4.6]$$

The form of $\rho_c(\sigma)$ is plotted in Figure 4.2, and the minimum at $\rho_c^{\min} = 27$ when $\sigma = 1$ indicates that phase coexistence occurs most readily when $K_m \approx I_{50}$; i.e., relatively low values of resistance efficacy ρ already give rise to coexistence. For the systems investigated in the experiments, σ is of order one and coexistence is predicted to occur for $\rho \gtrsim 29$ (see I_{50} and K_m values in Tables C.2, C.5). For the Cm-CAT system, this corresponds to a critical value of $(V_0 / \kappa)_{crit} \cong 350 \mu\text{M}$ based on the parameters in Table C.2.

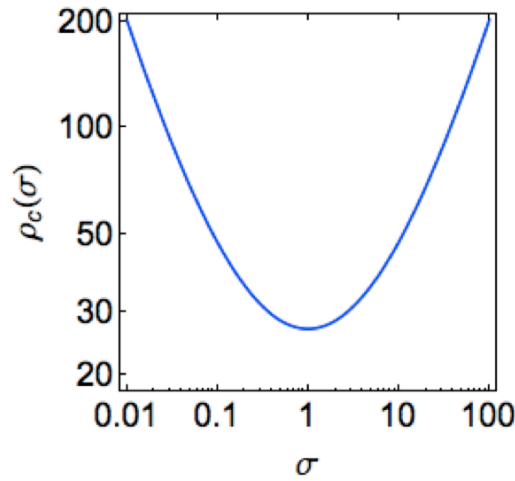


Figure 4.2. Criteria for growth bistability: the critical resistance efficacy

The critical resistance efficacy $\rho_c(\sigma)$ gives the minimal resistance efficacy required ($\rho > \rho_c(\sigma)$) in order for a strain to exhibit bistability over any range of drug concentrations.

4.2.3 The critical external antibiotic concentration

Similarly, solving Eq. [4.6] for $a_{i,c}$ gives

$$a_{i,c} = \sigma^{-1/3} + \sigma^{-2/3} \quad [4.7]$$

yielding the critical external concentration at the bifurcation point

$$a_{e,c} = \left(1 + \sigma^{-1/3}\right)^3 \quad [4.8]$$

or

$$[a]_{\text{ext}}^{\text{crit}} = \left(\sqrt[3]{I_{50}} + \sqrt[3]{K_m}\right)^3 \quad [4.9]$$

which takes the value $[Cm]_{\text{ext}}^{\text{crit}} \approx 67 \mu\text{M}$ for the Cm-CAT system (see Table C.2).

For $\rho < \rho_c(\sigma)$, there is a unique solution a_i for each external concentration a_e , and a homogeneous growth phase is expected. For $\rho > \rho_c(\sigma)$, $a_e^{\max}$ and $a_e^{\min}$ exist and correspond to the MIC and MDC respectively in the sense discussed below. I next describe the dependencies of MIC and MDC on ρ , which are readily probed in experiments by changing the basal expression level of antibiotic resistance (V_0 in the model).

4.3 Predicting MIC and MDC

4.3.1 Interpretation and prediction of the Minimum Inhibitory Concentration

(MIC)

To solve for the MIC, I look for the maximum of a_e as defined by Eq. [4.1]. The value of a_i giving the maximum, a_i^* (as illustrated in Figure 4.1B), is found to obey the

following equation,

$$\sigma \cdot (a_i^*)^2 = 1 + (1 + a_i^*)^2 \cdot (1 + \sigma a_i^*)^2 / \rho. \quad [4.10]$$

Solving for a_i^* in powers of $1/\rho$ (and identifying a_i^* as the smaller of the two solutions),

I obtain

$$a_i^* \approx \frac{1 + b/\rho}{\sqrt{\sigma}} \quad [4.11]$$

where $b = (1 + \sigma^{-1/2})^2 \cdot (1 + \sigma^{1/2})^2 / 2$, giving

$$a_e^{\max} \approx \frac{\rho}{(1 + \sigma^{1/2})^2} + \sigma^{-1/2} + \frac{1}{\rho} \frac{(1 + \sigma^{1/2})^4}{2\sigma^{3/2}} + \dots \quad [4.12]$$

from Eq. [4.1]. Since $\rho_c \geq 27$, then for $\rho > \rho_c(\sigma)$ where the maximum exists, the sub-leading terms in $1/\rho$ are negligible; see Figure 4.3A that illustrates this for the Cm-CAT system ($\sigma \approx 0.46$, Table C.2).

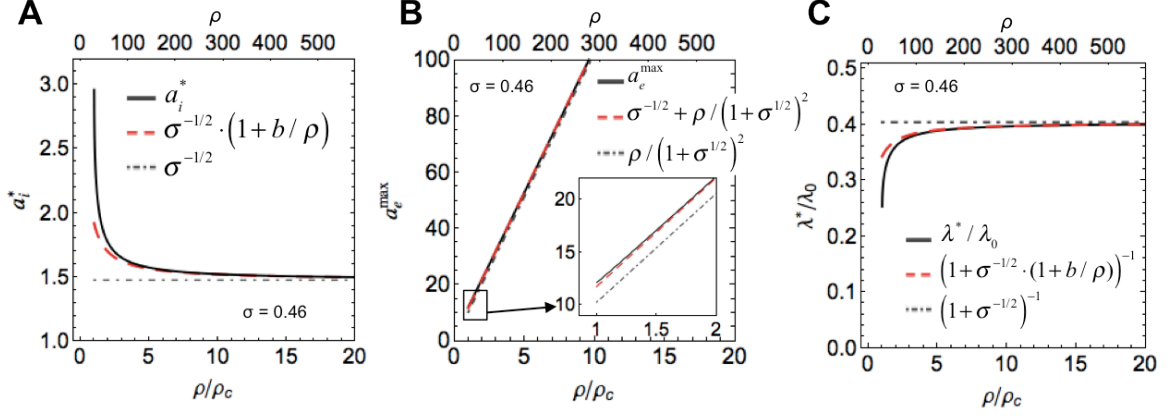


Figure 4.3. Prediction of the minimal inhibitory concentration (MIC)

A, The predicted internal antibiotic concentration a_i^* for cells growing in antibiotic medium just below the MIC, expressed as a function of resistance efficacy in absolute dimensionless units (top axis), or in units of critical resistance efficacy $\rho_c(\sigma)$. It is obtained as the ArgMax of equation [4.1]. **B**, The plot shows how $a_e^{\max}$ varies with resistance efficacy. $a_e^{\max}$ is the highest external antibiotic concentration to allow growth in a strain with strong resistance, such that $\text{MIC} \approx I_{50} \cdot a_e^{\max}$ and provides the upper bound for the region of bistability. **C**, The growth rate λ^* at $a_e^{\max}$ gives the minimum growth rate achievable in sub-inhibitory concentrations (i.e. for $[a]_{\text{ext}}$ just below MIC), and its value is independent of resistance efficacy for strains with strong resistance. Quantities are given for the Cm-CAT resistance system with saturability parameter $\sigma = I_{50}/K_m \approx 0.46$; see Table C.2 for details.

Thus, the MIC is predicted to be

$$\text{MIC} = a_e^{\max} \cdot I_{50} \approx \frac{V_0 / \kappa}{\left(1 + \sqrt{K_m / I_{50}}\right)^2} + \sqrt{K_m \cdot I_{50}} \quad [4.13]$$

which becomes, for the Cm-CAT system

$$\text{MIC} \approx 0.16 \cdot V_0 / \kappa + 8 \mu\text{M} \quad [4.14]$$

We compare these predictions to experimental results in section 4.4.

4.3.2 Criteria for dormancy and the Minimum Dormancy Concentration (MDC)

To solve for the MDC, I look for the minimum of Eq. [4.1], $a_e^{\min}$, which occurs at the internal concentration $a_i = a_i^\dagger$ (see Figure 4.1B for illustration). It is also necessary to establish the conditions on parameters ρ and σ for which growth is effectively completely inhibited when $a_i \geq a_i^\dagger$ (such that the system has a dormant state in addition to the growing state). I obtain $a_i^\dagger$ as the larger solution to Eq. [4.10]. In the limit $[a]_{\text{int}} \gg K_m$, or equivalently $a_i \gg \sigma^{-1}$, I find

$$a_i^\dagger \approx \sqrt{\frac{\rho}{\sigma}} \cdot \left(1 - \frac{\sigma^{1/2} + \sigma^{-1/2}}{\rho^{1/2}} \right) \quad [4.15]$$

to the first sub-leading order in $\rho^{-1/2}$, with the value of the minimum given by

$$a_e^{\min} \approx \sqrt{\frac{\rho}{\sigma}} \left(2 - \frac{\sigma^{1/2} + \sigma^{-1/2}}{\rho^{1/2}} \right) \quad [4.16]$$

This approximation is in good agreement with the full solution of $a_e^{\min}$ as shown in Figure 4.4A-B for $\sigma = 0.46$ (for the Cm-CAT system).

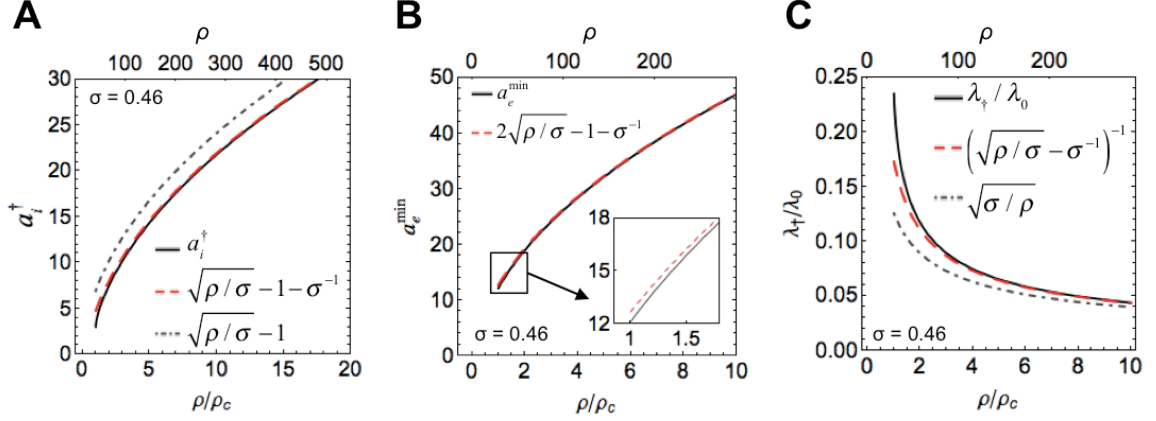


Figure 4.4. Prediction of the minimal dormancy concentration (MDC)

A, For a strain of efficacy ρ , $a_i^{\dagger}$ gives the minimum internal antibiotic concentration possible at steady state along the “low-growth” branch of the bistable region. It is obtained as the ArgMin of equation [4.1] and increases with the square root of resistance efficacy. **B-C**, The external antibiotic concentration for a cell with $a_i = a_i^{\dagger}$ is given by $a_e^{\min}$ and provides the lower boundary of the bistable region. This quantity also gives the MDC in the appropriate limit (see discussion surrounding Eq. [4.17]), i.e. when the growth rate at this point $\lambda_{\dagger}$ is small (given in **C**).

To determine whether or not a non-growing state exists for $a_e > a_e^{\min}$, I find the predicted growth rate at this boundary ($a_i = a_i^{\dagger}$)

$$\lambda_{\dagger} = \frac{\lambda_0}{1 + a_i^{\dagger}} \simeq \frac{\lambda_0}{\sqrt{\rho/\sigma} - \sigma^{-1}} \quad [4.17]$$

see Figure 4.4C. The growth rate takes on low values, $\lambda_{\dagger}/\lambda_0 < 0.1$ (typically too low to measure reliably in our experiments), for antibiotic resistant strains in this work except for parameters very close to the critical point. When $\sigma \gtrsim 2$ (and $\rho > \rho_c(\sigma)$ to ensure coexistence) this predicted growth rate can be appreciably greater than zero and is approximately

$$\frac{\lambda_{\dagger}}{\lambda_0} \approx \sqrt{\frac{\sigma}{\rho}} = \sqrt{\frac{I_{50} \cdot \kappa}{V_0}} \quad [4.18]$$

Equation [4.18] suggests that growth rate along this “slow growth” branch can be increased arbitrarily by decreasing the ratio ρ/σ ; however, bistability disappears for $\rho < \rho_c(\sigma) = (\sigma^{-1/3} + 1 + \sigma^{1/3})$ by Eq. [4.6]. The growth rate at $a_i = a_i^\dagger$ with $\rho = \rho_c(\sigma)$ gives the maximum value $\lambda_\dagger$ may take at $a_e^{\min}$:

$$\lambda_\dagger^{\max}(\sigma) = \frac{\lambda_0}{1 + \sigma^{-1/3} + \sigma^{-2/3}} \quad [4.19]$$

(with $\rho = \rho_c(\sigma)$). This value is small (e.g. $\lambda_\dagger^{\max} \leq \lambda_0/3$) when $\sigma \leq 1$, or equivalently when $I_{50} \leq K_m$. The I_{50} and K_m parameters for the CAT and TetA systems used in this study (corresponding to $\sigma = 0.46$ and 0.4 respectively), give $\lambda_\dagger^{\max}/\lambda_0 \approx 0.25$ at the critical point such that cell growth in the “high” $[a]_{\text{int}} > I_{50} \cdot a_e^{\min}$ branch of drug concentration is virtually completely inhibited. Thus, the minimal coexistence concentration $a_e^{\min}$ corresponds practically to the Minimal *Dormancy* Concentration (MDC), and $a_e^{\max}$ corresponds to MIC, as claimed above (Eq. [4.13]). Using the approximate form Eq. [4.16] and with $\rho/\sigma \gg 1$, the MDC is predicted to increase with the square root of drug resistance activity (V_0), as

$$\text{MDC} = a_e^{\min} \cdot I_{50} \approx 2 \cdot \sqrt{I_{50} \cdot V_0 / \kappa} - (I_{50} + K_m). \quad [4.20]$$

4.3.3 Predicting MDC based on MIC alone

Additionally, I can write the MDC in terms of MIC for strains with high levels of resistance. In the limit $\text{MIC} \gg \sqrt{I_{50} \cdot K_m}$, MIC becomes approximately proportional to V_0 / κ (to zero order in $\sqrt{I_{50} \cdot K_m} / \text{MIC}$, Eq. [4.13]). Then with Eq. [4.20] one finds a simple expression for MDC in terms of MIC

$$\text{MDC} \approx 2 \cdot \left(\sqrt{K_m} + \sqrt{I_{50}} \right) \cdot \sqrt{\text{MIC}} \quad [4.21]$$

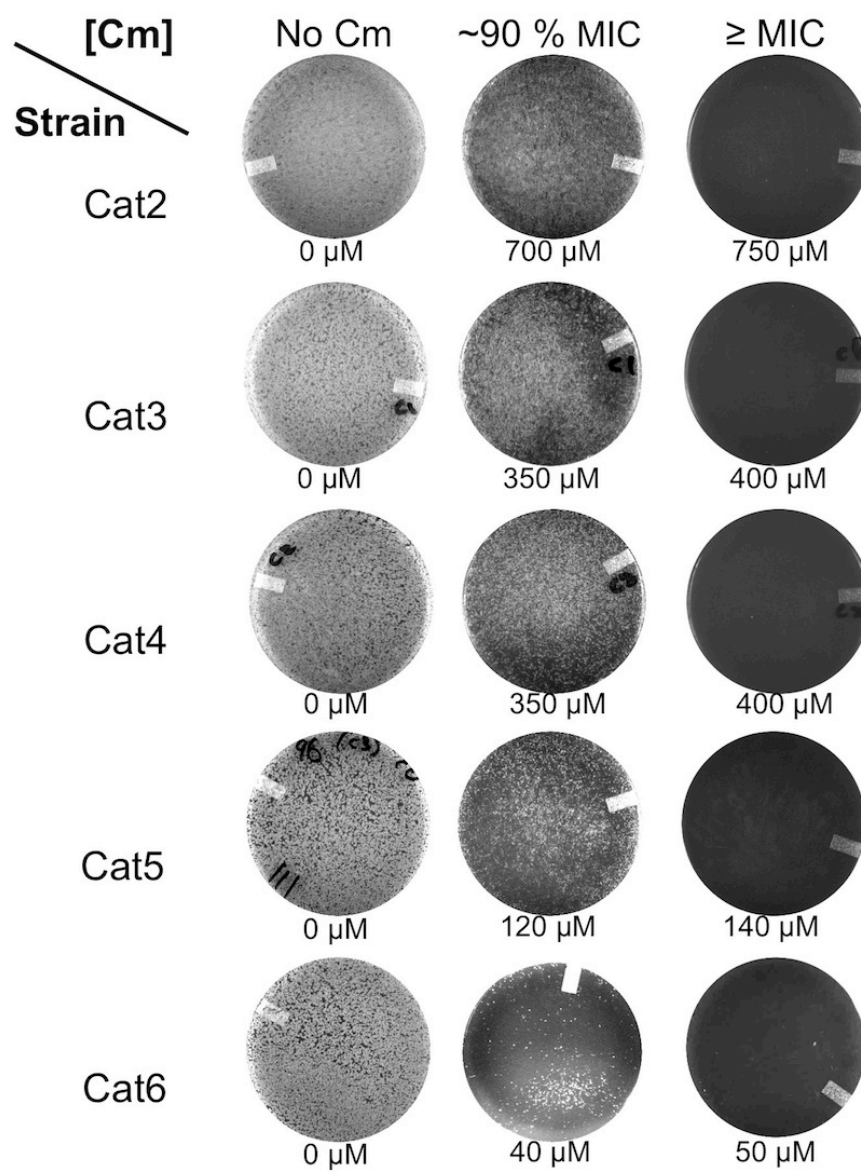
Thus, it is possible to predict the range of concentrations over which growing and non-growing cells coexist without explicitly knowing or fitting the value of parameter V_0 / κ . This, of course, is useful because one may not be able to measure V_0 / κ directly.

4.4 Experimental and theoretical growth bistability phase diagram

The results above predict the expected growth behaviors (MIC and MDC) of Cm-resistant strains across a 2D parameter space: Cm concentration versus basal CAT activity V_0 / κ . In addition to strain Cat1, five additional CAT-expressing strains (Cat2 through Cat6; Table C.1 and Table C.3) were designed to provide reduced degrees of constitutive CAT expression relative to strain Cat1. I measured the $\text{MIC}_{\text{plate}}$ for each of the strains; the results are shown in Figure 4.5.

Figure 4.5. Determination of MIC_{plate} for various CAT-expressing strains.

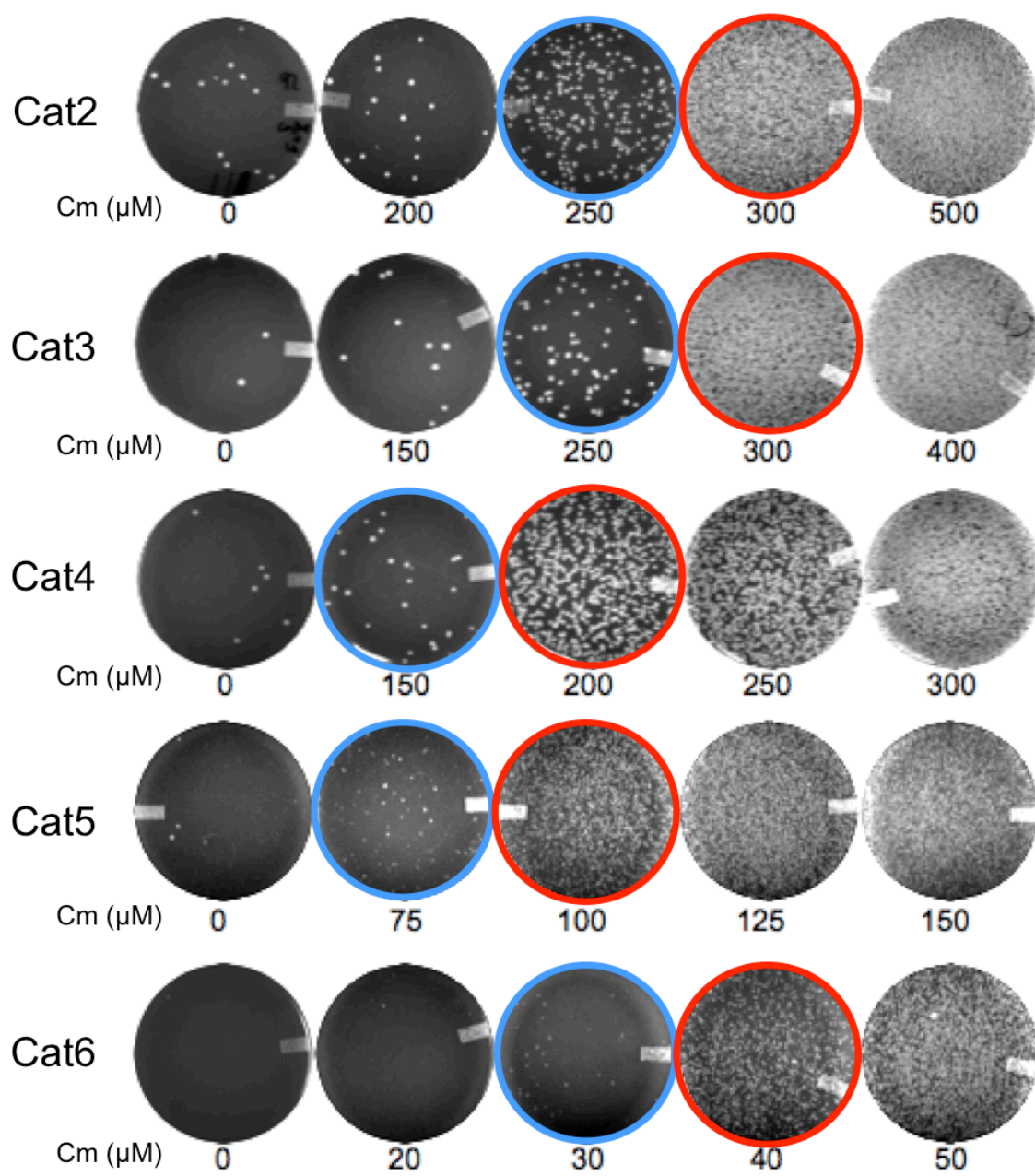
These results identify MIC_{plate} values of strains Cat2 through Cat6 (Table C.1); they are similar to those presented in Figure 2.3, but each strain harbors a unique level of constitutive CAT expression. For each strain shown, cells were diluted from log phase batch cultures lacking Cm and spread onto agar plates at a density of $\sim 8 \times 10^4$ cells per plate. Plates incubated overnight at 37°C . Chloramphenicol concentrations are indicated underneath each plate, and additionally as percentage of MIC_{plate} for each strain (top row). MIC_{plate} is taken as the Cm concentration above which colonies appear at a frequency of less than $\sim 10^{-4}$. At least two replicate experiments were performed for each strain; representative results are shown here. MIC_{plate} values were similar to those obtained with batch culture growth in minimal media with chloramphenicol (it is not uncommon to find MIC of an antibiotic in LB to correspond with MIC in minimal media (Bollenbach et al., 2009). For the data plotted in Figure 4.7, I used only MIC data determined in batch cultures with minimal media because the model is formulated based on observations during batch culture growth (see discussion in sections 3.2-3.5). The results in this figure are intended to complement the batch culture MIC determinations used in Figure 4.7.



I measured the MDC for strains Cat2-Cat6 using the same batch-culture enrichment procedure carried out as described in section 2.5. Each strain was enriched in Amp and various concentrations of Cm at and below the respective MIC for the strain. The results are shown in Figure 4.6, with CFU per inoculum revealing the relative fraction of cells surviving Amp enrichment for each Cm concentration, i.e. the dormant fraction of the population during the enrichment procedure.

Figure 4.6. Determination of MDC for various CAT-expressing strains.

The Amp enrichment assay (Figure 2.9) was performed for CAT-expressing strains Cat2 through Cat6 to identify the MDC of each strain, representative plate results are shown here. Plates circled in red indicate that at least several percent of Amp-treated cells formed colonies, while those circled in blue indicate <1% of colony formation. The MDC value for each strain in the phase diagram of Figure 4.7 gives the average of at least two experiments strain (except for strain Cat4, for which I only performed the enrichment experiment once).



Because the model is based on observations from growth in batch cultures, I also measured the MIC in batch culture for each strain (see 3.8.2.4 for details); MIC values obtained this way were largely consistent with the $\text{MIC}_{\text{plate}}$ values obtained from Figure 4.5. These batch culture MIC and MDC values are plotted in Figure 4.7 (strains as numbered circles and diamonds respectively) as a function of the CAT activity V_0 measured for each strain (gray bars, bottom of Figure 4.7). Assuming that the permeability κ does not differ significantly across these strains, the measured CAT activities give V_0/κ for all strains (relative to that of Cat1), as shown by the grey arrows in Figure 4.7. The empirical results are plotted on top of the “phase diagram” arising from the model: the expressions for MDC and MIC derived above in section 4.3 give the predicted regions of uniform growth, bistable coexistence, and uniform dormancy. The MIC (red line) is predicted to increase linearly with V_0/κ , while the MDC (blue line) is predicted to increase as $\sqrt{V_0/\kappa}$ (Eqs. [4.13] and [4.20] respectively). These two lines define a wedge in the parameter space of $[a]_{\text{ext}}$ and V_0/κ throughout which growth bistability is expected, terminating at a bifurcation point (purple point in inset) below which a uniformly growing population is predicted (see Eq. [4.9]). The model predictions capture experimental observations well except close to the bifurcation point (e.g., in strain Cat5, inset), without adjusting any parameters.

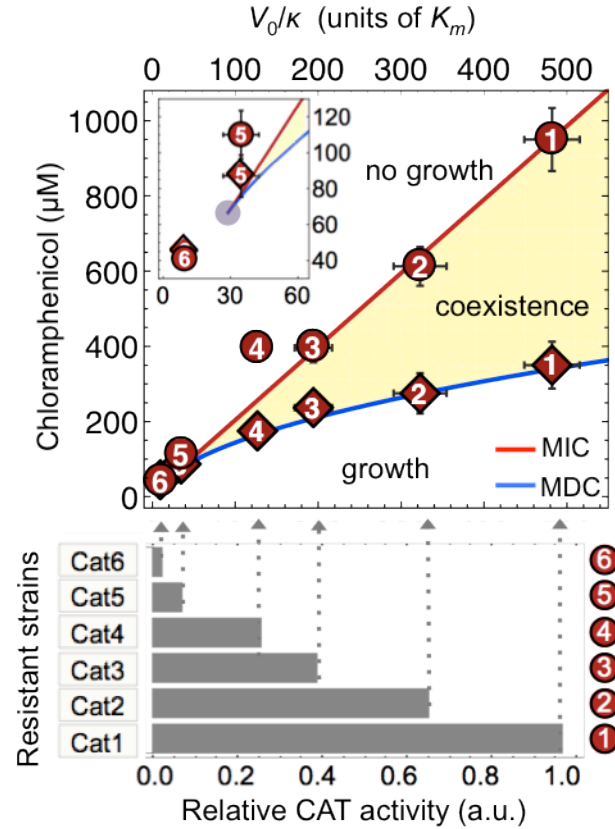


Figure 4.7. The phase diagram of growth bistability in the Cm-CAT system.

The MDC (blue line) and MIC (red line) predicted by the growth feedback model for strains with different degrees of basal CAT expression (V_0) define a phase diagram, with the coexistence of growing and dormant populations between the MDC and MIC (beige). The region of coexistence terminates at the critical point (purple point, inset) described in section 4.2 of the text. MIC determined in batch cultures (circles, procedure described in section 3.8.2.4; see also Figure 4.5) and MDC (diamonds, Figure 4.6) are measured for strains differing only in their levels of constitutive CAT expression (quantified by the relative CAT activity in the absence of Cm; see bar graph below). Error bars in MDC are estimated standard uncertainties due to experimental procedures (e.g., number and distribution of discrete Cm concentrations tested, pipetting error, etc.), and are equal to or larger than the range of MDC values obtained in independent observations such as those shown in Figure 4.6. Error bars in MIC are estimated standard uncertainties from experimental procedures, and are equal to or larger than the range of MIC values obtained in $n \geq 2$ independent observations in batch cultures. Additionally, the MICs determined on agar plates (called MIC_{plate} , see Figure 2.3, Figure 4.5 and methods in section 2.7.3.2) and in the microfluidic device (Figure 2.6) generally agreed with these values.

I mention briefly that the full growth model is formulated based on steady-state results, and as such, it can make no statement regarding the probability for any particular cell to either grow or remain dormant. I tried to choose the experimental conditions carefully to observe the full range of growing and dormant behaviors, but in fact the results reveal that stochasticity is important. For example: if the system were completely deterministically bistable, then a Cm-upshift from media without drug to media with Cm should yield only growing populations throughout the bistable region. However, we frequently saw heterogeneous responses during these types of experiments. It is likely that just prior to these shifts, cells exist in a mixture of physiological states that will influence their individual responses to a transient antibiotic upshift, but that these states have no noticeable effect in absence of antibiotic (e.g. cell permeability to the drug may vary across the population for whatever reasons, but this would not affect normal growth). While the kinetics of response to a transient antibiotic shock are interesting, they are beyond scope of this model and will likely depend critically on environmental factors and cell history.

4.5 Generalizations of the model and applicability to other drugs: a detailed study of tetracycline resistance

4.5.1 The effect of endogenous drug efflux and drug chelation on resistance

I formulated the model with the goal of describing effects of antibiotics on bacterial growth in the presence of a single high affinity efflux- or enzyme-mediated drug resistance mechanism. But as described near Eq. [3.2], cells often additionally possess

low-affinity native efflux mechanisms that may potentially confound this simple description. Additionally, some antibiotics may be charged by chelators under physiological conditions, ultimately affecting permeation rate and equilibrium distributions of the drug across cell membranes (Nikaido & Thanassi, 1993). I briefly treat the effects of endogenous efflux and chelation here, emphasizing that key results do not differ qualitatively from those derived above.

4.5.2 Modeling drug influx in the presence of endogenous drug efflux and drug chelation

We begin by incorporating the effects of chelation or endogenous efflux into the description of passive influx of drug into the cell. I assume the presence of low-affinity efflux or chelation alters the equilibrium concentration of internal drug by a factor of η such that $[a]_{\text{int}} = \eta \cdot [a]_{\text{ext}}$ at equilibrium. When η characterizes low-affinity efflux, comparison with Eq. [3.4] reveals that $\eta \equiv \left(1 + V_{\text{native}}^{\text{max}} / \kappa \cdot K_{\text{native}}\right)^{-1}$, and $\eta \leq 1$. If the parameter characterizes chelation, then η may take any positive value, indicating that Donnan potential may act to either increase ($\eta > 1$) or decrease ($\eta < 1$) internal drug concentration relative to extracellular concentration. One can thus describe the influx by modifying Eq. [3.2] to the form

$$J_{\text{influx}} = \kappa \cdot ([a]_{\text{ext}} - [a]_{\text{int}} / \eta). \quad [4.22]$$

Yet another layer of detail is that equilibrium may be shifted (as described above)

separately across the inner and outer membranes, giving two shift factors: η_{per} and η_{cyt} . For example, an endogenous efflux pump could expel antibiotic from the cytoplasm into the periplasm (which would be described by η_{cyt}), and this would have no effect on the equilibrium distribution of the drug between the periplasm and external medium (described by η_{per}).

4.5.3 Effects of endogenous drug efflux and drug chelation on phase coexistence, MIC, and MDC

Without providing the algebraic details here, I affirm that one can recover the steady state relation Eq. [4.1] by balancing influx across cell membranes with a high-affinity efflux term J_{removal} , and then rescaling variables judiciously. All subsequent derivations proceed as before, and one can show that in analogy to Eq. [4.6],

$$\rho_c(\sigma, \eta_{\text{per}} \cdot \eta_{\text{cyt}}) = \frac{(\sigma^{-1/3} + 1 + \sigma^{1/3})^3}{\eta_{\text{per}} \cdot \eta_{\text{cyt}}} \quad [4.23]$$

And in place of Eq. [4.13] one obtains

$$\text{MIC} \approx \frac{V_0 / \kappa_\eta}{\left(1 + \sqrt{K_m / I_{50}}\right)^2} + \frac{\sqrt{K_m \cdot I_{50}}}{\eta_{\text{per}} \cdot \eta_{\text{cyt}}} + \dots \quad [4.24]$$

with high-affinity efflux described by V_0 as before, and κ_η as effective permeability that generally depends on η_{per} and η_{cyt} . I showed above that effective permeability κ is defined by flux balance and depends on details of efflux (Eqs. [3.12], [3.13]) and κ_η must be defined in the same manner. The essential qualitative results of the model have

not changed; though this analysis shows that, for example, endogenous low-affinity efflux ($0 < \eta < 1$) would shift the critical point to a higher value (delaying the onset of bistability), but would not affect the linear relation between MIC and V_0 . And in analogy to Eq. [4.21], the theoretical MDC is given

$$\text{MDC} \approx 2 \cdot \left(\sqrt{K_m} + \sqrt{I_{50}} \right) \cdot \sqrt{\frac{\text{MIC}}{\eta_{\text{cyt}} \cdot \eta_{\text{per}}}} \quad [4.25]$$

Chloramphenicol is electrically uncharged at physiological pH and believed to move passively across the cell membranes (L.M. McMurry et al., 1994), and I can regard

$$\eta_{\text{per}} \approx \eta_{\text{cyt}} \approx 1.$$

4.5.4 Modeling tetracycline efflux by TetA in the presence of Tc-chelation

Tc is chelated by Mg^{2+} ions that strongly affect its partition, with $\eta_{\text{cyt}} \approx \eta_{\text{per}} \approx 2$. The equilibrium cytoplasmic tetracycline concentration is thus four-fold higher than the external concentration (Thanassi et al., 1995). In spite of this effect, I am justified in determining V_0/κ_{Tc} using Eq. [4.13] rather than Eq. [4.24] (Table C.5), because with $\text{MIC} \approx 700 \mu\text{M}$ (Figure 3.8A) and $\sqrt{K_m \cdot I_{50}} \approx 6.5 \mu\text{M}$ (Table C.5), I have $\text{MIC} \gg \sqrt{K_m \cdot I_{50}}$ and Eq. [4.24] is essentially equivalent to Eq. [4.13] with the definition $\kappa_{\text{Tc}} \equiv \kappa \cdot \eta_{\text{per}}$ (for TetA efflux, flux balance analysis reveals $\kappa_{\eta} = \eta_{\text{per}} \cdot \kappa = \eta_{\text{per}} \cdot (A_i \cdot \pi_{I.M.}) / \Omega_{\text{cyt}}$, independent of η_{cyt}).

Because $\eta_{\text{cyt}} \approx \eta_{\text{per}} \approx 2$ for Tc, Eq. [4.25] predicts MDC values for the drug-

resistant strain Ta1 to be lower (relative to MIC) than that predicted by Eq. [4.21]. However, high levels of Tc (and other antibiotics) have been shown to depolarize cell membranes (Gauthier, St-pierre, & Villemur, 2002; Novo, Perlmutter, Hunt, & Shapiro, 2000), so the shift factors likely have growth rate-dependence for which $\eta \rightarrow 1$ when Tc concentrations are high enough to completely inhibit growth. Thus, MDC in these systems may take some intermediate value between that given by Eq. [4.21] and that by Eq. [4.25]. Nonetheless, the plots in Figure 3.8A-B depict the region of bistability predicted by the analysis above with $\eta_{\text{cyt}} = \eta_{\text{per}} = 2$ for all antibiotic concentrations.

4.5.5 Constitutive expression of drug resistance and other forms of growth-mediated feedback

In the analysis I assumed constitutive expression of drug resistance. Aside from being the simplest system to characterize, CAT is indeed most often expressed constitutively in Gram⁻ bacteria from Tn9 and elsewhere (Shaw, 1983). This mode of expression also occurs naturally in *integrons*, in which many unregulated resistance genes are placed under the control of a single high-activity constitutive promoter (Harbottle et al., 2006). Resistance genes in resistant clinical isolates have also been found to be under the control of constitutive promoters (Piddock, 2006). But for a protein whose expression is specifically regulated, e.g. at the transcriptional or translational level, the growth-rate dependence need not be linear as in Figure 3.4 (Bollenbach & Kishony, 2011; Klumpp et al., 2009). In the simplest approximation one can modify Eq. [3.25] to the form

$$V_{\max} = V_0 \cdot (\lambda / \lambda_0)^n, n \geq 0. \quad [4.26]$$

Deriving the consequences of this relation is beyond the scope of the current work, but I mention here some key results: First, growth bistability and abrupt drops in fitness still occur for a broad range of the (ρ, σ) -parameter space, but the criterion Eq. [4.6] for the occurrence of the critical point depends on the value n . With the modification in Eq. [4.26], one can show that $a_e^{\max}$ still increases linearly with resistance efficacy ρ for strong resistance, and hence approximately $\text{MIC} \sim V_0 / \kappa$ as before, although the slope and intercept in Eq. [4.13] will depend nontrivially on n also. Interestingly, linear dependence of the empirical MIC on V_0 has also been reported for bacterial resistance to other antibiotics (Nikaido & Normark, 1987; Nordström, Ingram, & Lundbäck, 1972). This is likely a result of the generic relationship between $[a]_{\text{int}}$ and $[a]_{\text{ext}}$, in which Michaelis-Menten kinetics of resistance activity (Eq. [3.5]) are balanced by diffusive influx (Eq. [3.2]). Then, assuming cell growth is inhibited at some characteristic internal drug concentration (corresponding empirically to $[a]_{\text{ext}} = \text{MIC}$), simple flux balance dictates a linear relationship between this $[a]_{\text{int}}$ and MIC (Eq. [3.5], $[a]_{\text{ext}} \gg V_0 / \kappa$).

The MDC is more sensitive than MIC to the form of the feedback, with the result

$$\text{MDC} \sim (V_0 / \kappa)^{\frac{1}{n+1}} \quad [4.27]$$

For $n > 1$, the feedback is more cooperative and the MDC is reduced. Thus, the onset of growth bistability occurs at lower antibiotic concentrations as the cooperativity in feedback (n) is increased. Conversely, for weak feedback (in the limit $n \rightarrow 0$), the region

of bistability shrinks as $\text{MDC} \sim V_0 / \kappa$ approaches the MIC.

4.6 Predicting growth rates across multiple antibiotic-resistant strains

4.6.1 Predicting growth rates from enzyme activity

The CAT activities (V_0 / κ , bottom of Figure 4.7) can also be used to predict growth rate reductions (λ / λ_0) for these strains. The predictions are plotted together with the data (lines and circles of like colors) in Figure 4.8. The predictive power of the model is remarkable, as the lines are not fits to the data, but merely solutions to Eqs. [3.30] and [3.22] using the measured values of V_0 as input. Comparable agreements are obtained using the empirical MIC value for each strain (see section 4.6.2).

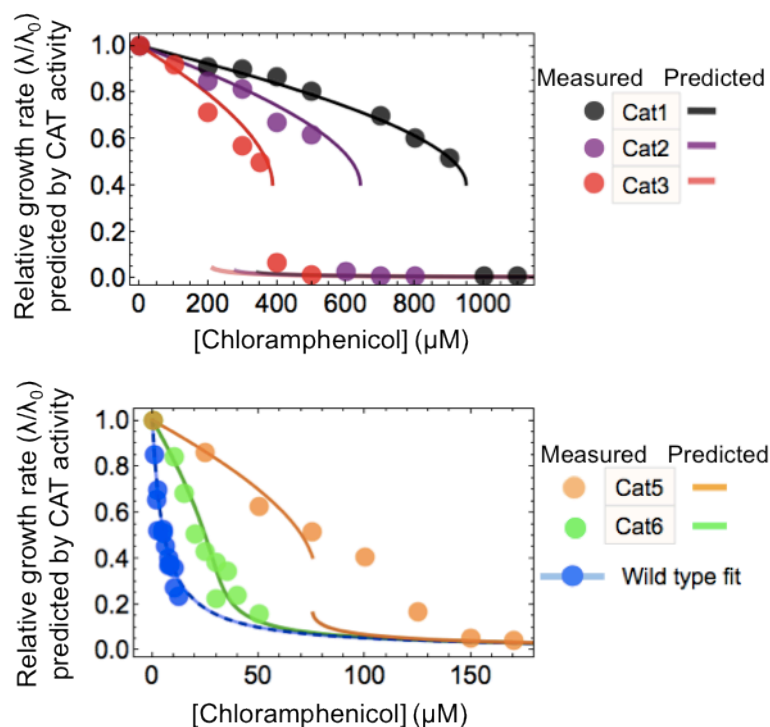


Figure 4.8. Predicting growth rates of Cm-resistant strains based on enzyme activity.

Measured and predicted growth rate (circles and lines of like colors), in minimal medium with varying Cm for strains of known relative CAT activities; the wild type is shown in blue for reference. Predictions were made with only the measured MIC for strain Cat1 and the relative CAT activity between the different strains, without any parameter fitting.

4.6.2 Predicting growth rates from MIC

As described above for strains Cat1 and Ta1 (Figure 3.7 and Figure 3.8), I can also use MIC alone to predict the growth rates of antibiotic-resistant strains by fixing V_0/κ with Eq. [4.13]. I show the MIC-based growth rate predictions for the CAT-expressing strains here in Figure 4.9. This can be a very useful tool because it provides growth rate predictions even if the particular resistance enzyme is unknown, or its activity is not easily assayable, e.g. in the Tc-TetA system, for which the activity of drug resistance (provided by the efflux pump) is not easily assayable.

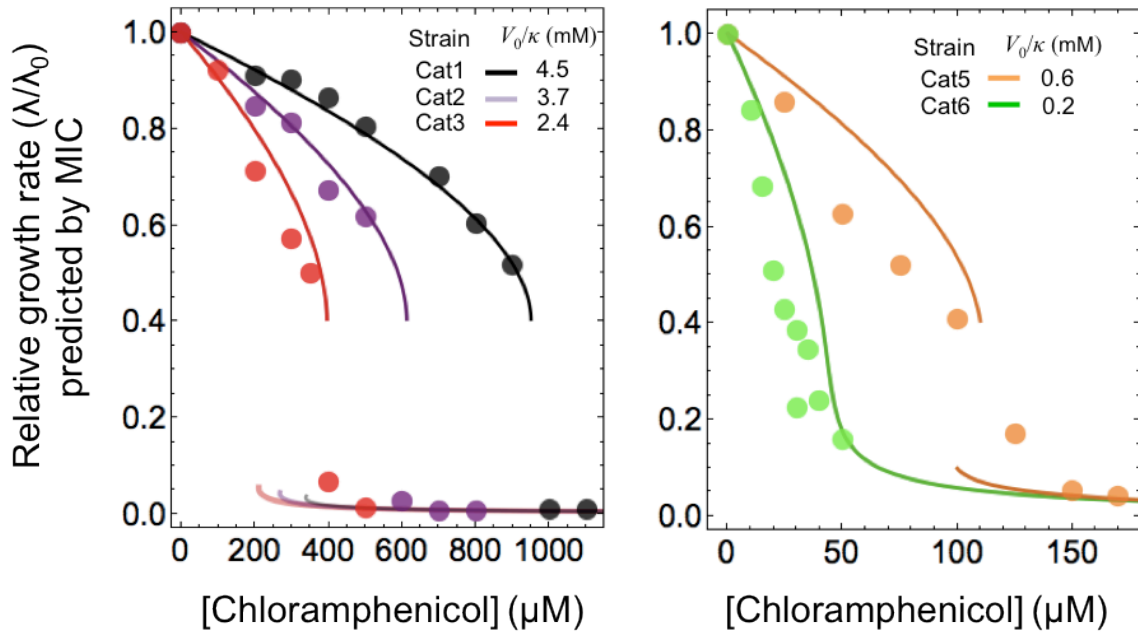


Figure 4.9. Growth rates predicted by the measured MICs in the Cm-CAT system.

To predict the growth rates of CAT-expressing strains in medium with various Cm concentrations (Figure 4.8), I used the value of V_0/κ determined by MIC for strain Cat1 together with the relative CAT activities (V_0) measured for the different strains (bottom of Figure 4.7). Here, I instead used only the empirical MICs of the CAT-expressing strains (circles in Figure 4.7) to fix the quantity V_0/κ for each strain with equation [4.13]. This procedure is able to predict growth rates even if resistance enzyme activity is not easily assayable, e.g. in the Tc-TetA system for which the activity of drug resistance (provided by the efflux pump) is not readily assayable but for which MICs are available.

4.6.3 A null hypothesis: performance of a model lacking growth-mediated feedback

Given the very nonlinear relation between the internal and external Cm concentrations for strongly resistant cells (Figure 3.2), I investigated whether a model without feedback, i.e., with a constant, growth-rate independent expression of CAT, might also fit the Cm-dependence of growth rate, with fitting parameter V_0/κ . Data from slow-growing or non-growing cultures ($\lambda/\lambda_0 < 0.15$) were excluded from the fitting

procedure to allow the no-feedback model the best possible chance to fit growth rates.

Figure 4.10 shows that the no-feedback model can reasonably account for growth data of strains Cat6 and Cat5, which exhibit little or no growth bistability, but the model fails to describe the growth data for the other strains that exhibit growth bistability and abrupt drops in growth rate, even at a qualitative level. If growth-mediated feedback did not play a role in growth, then Eqs. [3.6] and [3.21] would determine growth rate. With $V_{\max}$ as a constant, the growth rate is expected to decrease smoothly with increasing external antibiotic concentrations (dashed lines in Figure 4.10), similar to the response of wild-type cells.

To make a direct comparison between presence and absence of feedback, I include a similar fit for the growth feedback model using V_0/κ as the lone fitting parameter (solid colored lines), again excluding the non-growing cultures from the fit procedure; the latter clearly account for the data as a whole much better. The function used to fit the data was the implicit solution to equations [3.30]-[3.21]: $\lambda/\lambda_0([Cm]_{\text{ext}}; V_0/\kappa)$, with other parameters fixed (K_m and I_{50} , Table C.2). Note that these curves from the full model (solid lines of various colors) differ from the solid lines in Figure 4.8 because the latter are not fits; they are predictions of the model using the value of V_0/κ determined, respectively, from the measured MIC of Cat1 and the relative CAT activities of strains Cat2 through Cat6. Here I show the converse: that growth rates predict MIC reasonably well, despite the exclusion of non-growing cultures from the fitting procedure. This prediction is most striking for the strains with greatest CAT expression, in which growth rates alone accurately predict the location and approximate

magnitude of the abrupt drop in growth. See also Figure 4.9).

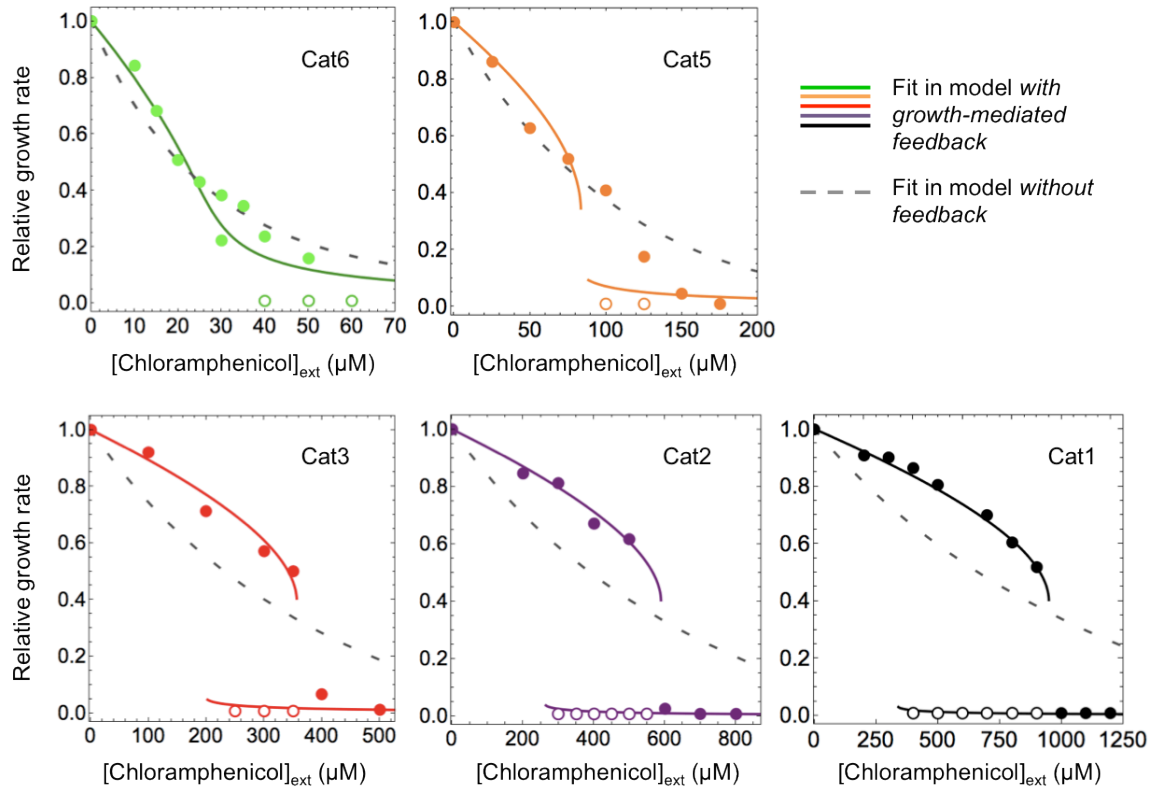


Figure 4.10. Growth model without drug-induced feedback.

We investigated whether a model without feedback, i.e., with a constant, growth-rate independent expression of CAT, might also explain the Cm-dependence of growth rate (data in Figure 4.8, reproduced here as solid circles, together with Amp-enrichment data shown as open circles). The no-feedback model (Eq. [3.6]) is used here to describe the data in this figure, using V_{max}/κ as the only fitting parameter. The best-fit model for each strain is shown as the dashed gray line. I fit the equation to the growth rates $\{[\text{Cm}]_{\text{ext}}, \lambda_i / \lambda_0\}$ using the Levenberg–Marquardt method with the “NonlinearFitModel” function in Mathematica; data from slow-growing or non-growing cultures ($\lambda/\lambda_0 < 0.15$) were excluded from the fitting procedure to allow the no-feedback model the best possible chance to fit growth rates. Including the non-growing data into fits produced qualitatively similar results.

4.7 Experimental materials and methods for Chapter 4

4.7.1 Strain construction

All of the strains used in this chapter are derived from *Escherichia coli* K12 strain MG1655 and described in Table C.1. DNA oligos used for construct and strain preparation are described in Table C.4. For all experiments, strains stocks were stored at -80 °C. Plates were streaked with stock strains within two weeks of use and stored at 4 °C.

4.7.2 Chromosomal incorporation of resistance markers

4.7.2.1 Construction of strain Cat2

Strain Cat1 was constructed as described in section 2.7.1.1. To make chromosomal *cat* driven by $P_{Ltet-O1}$ (Lutz & Bujard, 1997), the same *cat* gene as above was cloned into *KpnI/BamHI* sites downstream of $P_{Ltet-O1}$ in pKDT_ $P_{Ltet-O1}$ (Klumpp et al., 2009), yielding pKDT_ $P_{Ltet-O1}$ -*cat*. The cassette “ Km^r :*rrnBT*: $P_{Llac-O1}$ -*cat*” from this plasmid was incorporated into the same *ycaC/ycaD* site as described above. This strain is named EQ92, referred to as Cat2 elsewhere in the text.

4.7.2.2 Construction of strains Cat3-Cat6

As expected, the Cat1 cells with the chromosomal $P_{Llac-O1}$ -*cat* were highly resistant to Cm. To vary Cm resistance levels using site-directed mutagenesis, we

modified the region containing the ribosome binding site (RBS) in P_{Lac-O1} by inserting 4 nucleotides (TCCT) immediately downstream of RBS or/and changing one or two nucleotides in RBS (see Table C.3). The modified P_{Lac-O1} promoters (containing various altered RBS) together with *cat* were moved to the chromosome at the same location as described above. The resulting strains are referred to as Cat3 through Cat6.

4.7.3 CAT assays

The data in this chapter come from two separate CAT assays, the radiometric assay and the spectrophotometric CAT assay as described below. The spectrophotometric assay was used to obtain an estimate of the absolute CAT activity per OD_{600} in strain Cat1 (Table C.2) and Cat2, and horizontal error bars for CAT activities of strain Cat1 in Figure 4.7 are s.d. with $n=7$, and error bars for strain Cat2 give the range of values from $n=3$ independent measurements. We used the radiometric assay (performed on Cat1 and Cat3-Cat6) to determine the CAT activities of strains Cat3-Cat6 relative to Cat1. Error bars of CAT activities for strains Cat3-Cat6 in Figure 4.7 are estimated standard uncertainties from experimental procedures, and are equal to or larger than the range of values obtained in two independent assays. Relative CAT activities of all strains Cat1-Cat6 are given identically in the bottom of Figure 4.7.

4.7.3.1 Sample collection and cell permeabilization for CAT assays

We collected samples (at $OD_{600} \sim 0.15 - 0.4$) from cultures growing in minimal medium after at least 3 generations of exponential growth and stored them at $-80\text{ }^{\circ}\text{C}$ for assays the following day. To thaw samples we added an equal amount of extraction

buffer (0.2 M Tris, pH 8.0, 10 mM EDTA, and for the radiometric assay, an additional 40 mM β -mercaptoethanol). We then added toluene to 1% by volume, vortexed vigorously for 15 seconds (or 10 seconds for radiometric assay) and allowed cultures to incubate at room temperature for 15 minutes before performing the assays as described below (either spectrophotometric or radiometric). To identify the range in the assay over which CAT activity was proportional to CAT protein levels, we diluted samples with various volumes of permeabilized wild type cells (EQ4) and assayed activity. CAT activities reported in this study are within the linear dose-response window as identified above.

4.7.3.2 *Spectrophotometric CAT assays*

We used essentially the method of Shaw (Shaw, 1975). The assay was performed by aspirating 15 μ L permeabilized sample into 135 μ L reaction buffer inside the cuvette of a Thermo Scientific Genesys 20 spectrophotometer preheated to 37 °C. The standard reaction buffer contained 150 μ L of 4 mg/ml 5,5'-dithiobis-(2-nitro- benzoic acid) suspended in Tris/HCl, pH 8.0, 60 μ L each of 20 mM Acetyl-CoA and 5 mM Cm, and 1.08 mL deionized H₂O. We recorded absorbance A_{412} every 6 seconds using VISIONlite software (Thermo Scientific) between minutes 4-8 of the reaction (absorbance increased linearly during the reaction for at least 10 minutes); reported activity was taken as the slope of the absorbance over time divided by the final OD₆₀₀ of cells in the reaction volume (U/OD₆₀₀). To obtain the *in vitro* activity estimate for strain Cat1 (Table C.2), we performed a total of 7 assays from samples collected from two replicate cultures on different days with fresh media and reagents to obtain a value repeatable to within ~10%.

4.7.3.3 Radiometric CAT assays

Radiometric CAT assay was performed essentially as described by Seed and Sheen (Seed & Sheen, 1988). Briefly, after samples thawed they were diluted to OD₆₀₀ = 0.32 (Bio-Rad SmartSpec Plus) by the medium used for the culture. The assay was performed by adding 50 mL permeabilized sample to 75 mL reaction mixture preincubated at 37 °C for 5 min to give final concentrations of 100 mM Tris/HCl, pH8.0, 100 mM [3H] chloramphenicol (25 mCi/mmol), 240 mM butyryl CoA, 1% (vol/vol) ethanol. After incubation at 37°C for 30 min, the reaction was terminated by addition of 250 mL xylene, and mixed vigorously by vortexing. After centrifugation, 80% of the xylene phase was extracted twice with an equal volume of 10 mM Tris/HCl, pH7.5, 1 mM EDTA. The remaining organic phase was counted in a scintillation counter.

4.8 Acknowledgement

With permission from the coauthors, chapters 2-5 of this dissertation contain work from a manuscript that has been submitted for publication with the working title, “The innate growth bistability of antibiotic resistance,” 2013, Deris, JB; Kim, M; Zhang, Z; Okano, H; Hermesen, R; Groisman, A; Hwa, T. The dissertation author and Minsu Kim are co-first authors in the submitted work.

Chapter 5

Fitness landscapes and the evolution of antibiotic resistance

The evolution of antibiotic resistance is a complex problem in which human behavior and microecology combine to select for the molecular mechanisms conferring resistance. In the preceding chapters I described how those mechanisms determine the growth responses of drug-resistant bacteria to translation-inhibiting antibiotics. In this final chapter I show that these mechanisms lead generically to “plateau-shaped” fitness landscapes, and I briefly discuss the ways in which these fitness landscapes can be used to study the evolution of antibiotic resistance. In fact there is much to say on the topic, but the purpose of this final chapter is highlight to the significance of the preceding results in the context of evolution (and in particular, the importance of understanding the quantitative relationship between fitness, resistance phenotype, and environment). So here I will restrict the discussion to a few simple examples and the prerequisite introductory material.

5.1 Emergence of the plateau-shaped fitness landscape

In examining the growth rates of CAT-expressing strains in section 4.6 (e.g. Figure 4.9), it becomes apparent that in strains exhibiting an abrupt drop in growth, the growth rate just near the MIC is predicted to be the same for each strain, with a value $\lambda / \lambda_0 \approx 0.4$. In other words, the magnitude of the growth rate drop across the MIC (the “plateau height”) is approximately constant for all CAT-expressing strains that exhibit the abrupt behavior, apparently independent of the exact value of V_0/κ . I derive this basic result here.

To begin, recall that the internal antibiotic concentration (a_i) for cells growing in a near-MIC drug concentration (atop this growth plateau) is given by $a_i^* \approx \frac{1+b/\rho}{\sqrt{\sigma}}$ (Eq. [4.11]), with b as some function of σ . In Figure 4.3B, I show that Eq. [4.11] approximates the full solution for a_i^* well until very close to the critical point (compare the solid black and dashed red lines). In the limit $\rho \gg b$, which corresponds to $\rho \gtrsim 4 \cdot \rho_c(\sigma)$ for the Cm-CAT system, the leading-term approximation in Eq. [4.11] (dashed black line)

$$a_i^* \approx \frac{1}{\sqrt{\sigma}} = \sqrt{K_m / I_{50}} \quad [5.1]$$

already suffices. In this limit, Eq. [4.1] is reduced to

$$a_e \approx a_i \cdot \rho \cdot \frac{1}{1+a_i} \cdot \frac{1}{1+\sigma \cdot a_i} . \quad [5.2]$$

The growth rate at $a_e^{\max}$ becomes independent of ρ , with

$$\lambda^* = \frac{\lambda_0}{1 + a_i^*} \approx \frac{\lambda_0}{1 + \sqrt{K_m / I_{50}}}, \quad [5.3]$$

giving

$$\lambda^* \approx 0.4 \cdot \lambda_0 \quad [5.4]$$

for the Cm-CAT system (Figure 4.3C). As no growth is allowed for concentrations above MIC, λ^* represents the size of the abrupt drop in growth rate as the drug concentration is increased beyond the MIC. Growth rate data are largely consistent with the approximate independence of λ^* on the level of drug resistance activity V_0 as long as resistance efficacy $V_0 / (\kappa \cdot K_m)$ is not too close to the critical point rc ; see e.g., Figure 4.8.

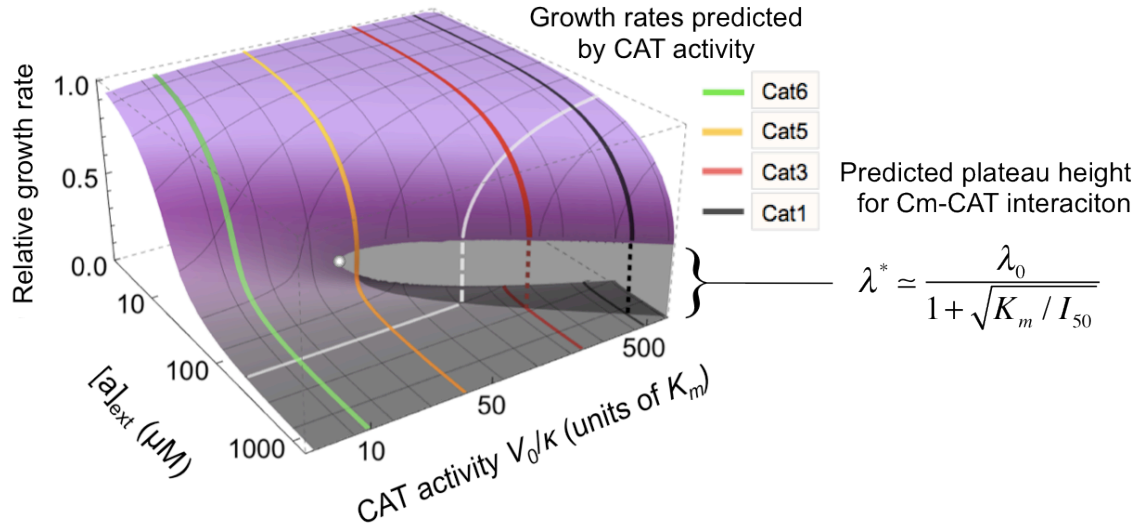


Figure 5.1. The fitness landscape of the Cm-CAT system.

Predicted growth rates (height of surface) for arbitrary CAT activity and Cm concentration (V_0 and $[a]_{\text{ext}}$ respectively): High (purple surface) and low growth rates (grey surface) overlap in the region of coexistence (growth bistability) that terminates at the bifurcation point (filled white ball). Predictions from Figure 4.8 are reproduced here (colored lines). The orthogonal white line illustrates the expected effect of changing CAT activity at a fixed Cm concentration; it can be viewed as a plateau-shaped fitness landscape.

Figure 5.1 gives the full solution of the model for strains with a range of CAT activity (V_0/κ) in medium with arbitrary Cm concentration ($[a]_{\text{ext}}$). The colored lines reproduce the predicted growth rates of several strains from Figure 4.8 and span a range of behaviors, from sub-critical to bistable. Viewing this plot orthogonally, the white line illustrates growth rates in an environment of fixed Cm concentration for strains of different CAT activities. As the CAT activity levels (V_0) are determined directly by molecular properties encoded by the genotype, e.g., the promoter or ribosomal binding sequences (Table C.3) and the coding sequence of the CAT gene, the white line describes a relation between the growth rate and the genotype, and may be regarded as a “fitness landscape”. There is such a fitness landscape for each environmental Cm concentration.

For $[a]_{\text{ext}} > [a]_{\text{ext}}^{\text{crit}}$ (Eq.[4.9]) these fitness landscapes are plateau-shaped, characterized by a threshold level of CAT activity across which the growth of the culture changes abruptly from non-growing to a value given by λ^* .

The emergence of a plateau-shaped fitness landscape from constitutive expression of drug resistance is surprising. It is a result of growth bistability, which arises from positive feedback due to global growth-dependent effects (Scott et al., 2010). As demonstrated here, the effect does not require special regulatory mechanisms or any molecular cooperativity, and it is not limited to a specific enzymatic mechanism of drug resistance. Therefore, I expect the growth bistability and the accompanying plateau fitness landscape to be robust features innate to drug-host interaction, at least for the class of translation-inhibiting antibiotics investigated here.

5.2 Defining fitness landscapes

I begin by discussing how the term “fitness landscape” as I will use it in this chapter differs from more common definitions of fitness landscapes, and how the two can be reconciled. Although the original axes in Wright’s landscape were intended to represent genotypes (Wright, 1932), the fitness landscapes described in Figure 5.1 took a phenotype as the independent variable (the resistance activity relative to the permeability, V_0 / κ) and gave growth rate as the measure of fitness. In doing so the fitness landscapes provide a precise mathematical mapping from phenotype to fitness that does not require detailed knowledge of genotype. (Genotype information is not always available and not always useful anyway. Below, in section 5.6, I briefly explore the how to use the

phenotype-based landscape here to predict the effects of genotypes on fitness.)

Under the standard definition of a fitness landscape, each environment requires a separate landscape for description, or else one must otherwise formulate some “average fitness over environments” to describe the contribution of a certain gene (Layzer, 1978). This issue is particularly relevant for our discussion of fitness in antibiotic resistance, in which the very trait under consideration is the microbial response to environmental harm. For this reason, the term “fitness landscape” in Figure 5.1 referred to the possible fitness values for a fixed concentration of C_m , as a function of the independent variable V_0/κ .

Furthermore, when considering drug resistance from a clinical perspective it's important to note that pharmacokinetic parameters vary between different organs and tissues that bacteria may infect, and that antibiotics are often administered periodically during treatment (Elliott, Beming, Iseman, & Peloquin, 1995; Meulemans, Paycha, Hannoun, & Vulpillat, 1989). As a result, antibiotic concentrations are not homogeneous across either space or time in the infection microhabitat (Baquero & Negri, 1997; Fry, 1996; Meulemans et al., 1989). In fact, recent theoretical and experimental results show that spatially heterogeneous environments (Greulich, Waclaw, & Allen, 2012; Hermesen, Deris, & Hwa, 2012; Zhang et al., 2011) can accelerate the emergence of antibiotic resistance, compared to the rate at which adaptation occurs in a homogeneous environment.

Therefore, because it is wholly inappropriate to consider fitness of antibiotic-resistant organisms in the absence of environment and because typical environments are naturally dynamic, it is natural to use the term fitness landscape to describe the fitness surface determined by the entire range of environment-cross-phenotype combinations.

Thus in this chapter a fitness landscape is defined as just such a surface (e.g., the entire 3-dimensional purple surface of Figure 5.1 on page 114). In this way, the fitness of a particular strain is characterized by a curve in the landscape that describes the strain's growth rates in all possible environments. With this definition I can discuss evolution within the context of these landscapes.

5.3 Mutations in a landscape

5.3.1 Size effect of mutations on phenotype

To discuss adaptation I must specify the way in which mutations change an organism's location within the fitness landscape. In the mathematical model of growth rate, the parameter V_0 / κ determines the strain's phenotype (and hence position in the landscape) and may take any positive real value. Although the phenotype is characterized by a continuous variable, mutations are discrete events that may bring about large quantitative changes in phenotype, even for single base pair substitutions—the smallest unit of genotypic distance. To illustrate, Figure 5.2 shows the locations of three CAT-expressing strains within the landscape specified by their measured in vitro CAT activities (Cat6, Cat5, and Cat3; blue, orange, and red).

These strains, in particular, make a useful example because they are “neighbors” in genotype space: a single nucleotide substitution in the RBS region upstream of the *cat* gene distinguishes strain Cat6 from Cat5, while another substitution in the same region distinguishes strain Cat5 from Cat3 (see Figure 5.2 and Table C.3). However, the value of V_0 / κ for strain Cat5 is ~5-fold different from the values of each of the other strains.

Thus, the two unit steps in genotype space from strain Cat6 to strain Cat3 are each accompanied by a ~ 5 -fold multiplicative increase in the value of the phenotype. Note that because $\text{MIC} \sim V_0 / \kappa$, MIC in this case also increases by a factor of 5 with each unit step in genotype space.

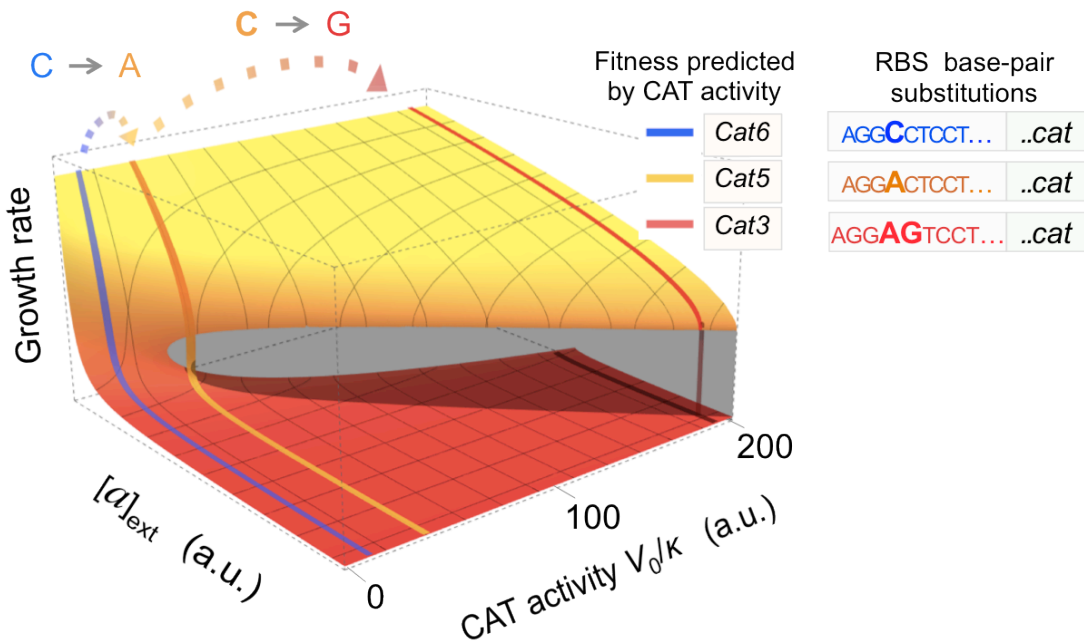


Figure 5.2. Discrete phenotypic jumps in a continuous-phenotype fitness landscape

The fitness landscape for the Cm-CAT system ($\sigma = \sigma_{\text{CAT}}$), as depicted in Figure 5.1 but shown here in linear scale, illustrates that strains that are genetically “near” one another in genotype space are not necessarily near one another in the phenotypic space of the fitness landscape. Each base pair substitution (gray arrows) results in a ~ 5 -fold change in CAT expression and MIC.

By examining how mutations affect a cell’s molecular properties, and consequently its physiological phenotypes, I can understand how a series of mutations would give rise to a multiplicative increase in MIC. And because MIC is proportional to the level of resistance protein activity according to Eq. [4.13], characterizing the effects

of mutation on gene expression will suffice. For example, gene amplification events are common during the development of antibiotic resistance (Sun, Berg, Roth, & Andersson, 2009), and every tandem duplication of a region containing a resistance gene would result in a two-fold increase in gene copy number (and hence MIC). Alternatively, point mutations in the gene's promoter region could increase gene expression by recruiting (or losing) some transcription factor (TF). Even if each mutation in the relevant region may only change the TF-DNA binding energy by some small amount, but TF-DNA binding probability depends exponentially on binding energy (Fields, He, Al-Uzri, & Stormo, 1997; Stormo & Yoshioka, 1991), and hence transcription rate can increase by a multiplicative factor with each mutation (Gerland, Moroz, & Hwa, 2002; Lässig, 2007). The basic arguments in the latter example have also been shown to apply to the relation between mRNA-ribosome binding energies and the translation initiation rate on a transcript, which explains the results in Figure 5.2. Therefore during the evolution of antibiotic resistance, one might expect that MIC also increase geometrically with the number of mutations.

5.3.2 *In vitro* evolution of antibiotic resistance

Indeed, the phenomenon of exponential increase in MIC over time during the evolution of antibiotic resistance has been well documented. In two studies carried out nearly 60 years apart, strains initially sensitive to chloramphenicol were exposed to increasing amounts of the drug over time: Cavalli and Maccacaro described that during each step of increased exposure, MIC increased by a factor of ~ 2 or less until drug

concentration in the media approached its solubility limit (L. Cavalli & Maccacaro, 1952). Much later, Toprak *et al.* reported a similar result in which MIC increased exponentially with time throughout a period of approximately a week before stalling at some upper limit of Cm concentration after two weeks of continuous dynamic exposure to the drug (Toprak et al., 2011). Both of these experiments involved prolonged exposure to concentrations of chloramphenicol that changed over time, and both readily produced mutants.

In Cavalli’s study, the authors attributed the large number of small evolutionary steps to the large number of possible mutation targets to increase Cm resistance (the MICs of backcrossed mutants support these claims). Interpreting this data in light of the current work suggests that the genotypes conferring resistance to Cm are well distributed throughout the phenotypic landscape presented above, as many mutations could affect the $V_{\max}$ of efflux (e.g., by gene duplications, promoter or RBS mutations, etc.) or the “effective” permeability defined in 4 (e.g. by changing the molecular composition of membrane or porin expression) (Hancock & Bell, 1988). In fact, beneficial mutations are more likely to affect the parameter V_0/κ than other parameters in the model: The parameter I_{50} is proportional to K_D (Eq. [3.22]), the dissociation constant describing drug-ribosome binding, and there are only a handful of residues at the binding site that interact directly with Cm (Dunkle, Xiong, Mankin, & Cate, 2010). Furthermore, most ribosomal mutations will likely be accompanied by the high fitness costs associated with ribosomal mutation in drug-free conditions (Lind, Berg, & Andersson, 2010). Recall that the Michaelis constant K_m sets the intracellular drug concentration at which enzyme activity reaches its maximum rate. Therefore, a mutation reducing the K_m to a value below the I_{50}

can allow resistance enzyme activity to reach its maximum rate even before growth is inhibited significantly. However, due to the paucity of plausibly beneficial single-step mutations at an enzyme's active site, the K_m is also unlikely to change considerably via mutation (Lewendon, Murray, Kleanthous, Cullis, & Shaw, 1988; Lewendon, Murray, Shaw, Gibbs, & Leslie, 1994; Murray, Lewendon, & Shaw, 1991).

With this information, modeling the adaptation to C_m is feasible with the fitness landscapes developed in this work. And one would be justified in assuming that, on average, V_0 / κ increases by some multiplicative factor with each mutation, even without modeling the effect each individual mutation separately.

5.4 The tale of two landscapes

According to Eq. [5.3] the height of the abrupt drop in the fitness landscapes, λ^* , depends on the combination of system parameters that I have called saturability (I_{50} / K_m , Eq. [4.3]), with $\lambda^* = \lambda_0 / (1 + \sqrt{K_m / I_{50}})$. Figure 5.3 shows two landscapes, each with very different plateau heights λ^* due to the difference in saturability. The fitness landscape on the left corresponds to small saturability (e.g., large K_m); it is the prototypical landscape for strains in which resistance is conferred by so-called “low-affinity” efflux ($K_m \geq 100 \mu\text{M}$) (Levy, 1992; L.M. McMurry et al., 1994). Evolution through this type of landscape is expected for strains that a) are not initially resistant to the drug (like strains in the in vitro evolution described in section 5.3.2) and b) do not obtain the relatively unlikely series of mutations required to greatly increase the saturability of the endogenous efflux proteins for the drug and hence significantly change

the landscape. This endogenous efflux landscape resembles a gently sloping hillside. Conversely, the landscape on the right illustrates fitness possibilities during evolution when an efflux enzyme of high saturability ($I_{50} \gg K_m$) confers resistance. This is an extreme example of a mesa-shaped fitness landscape.

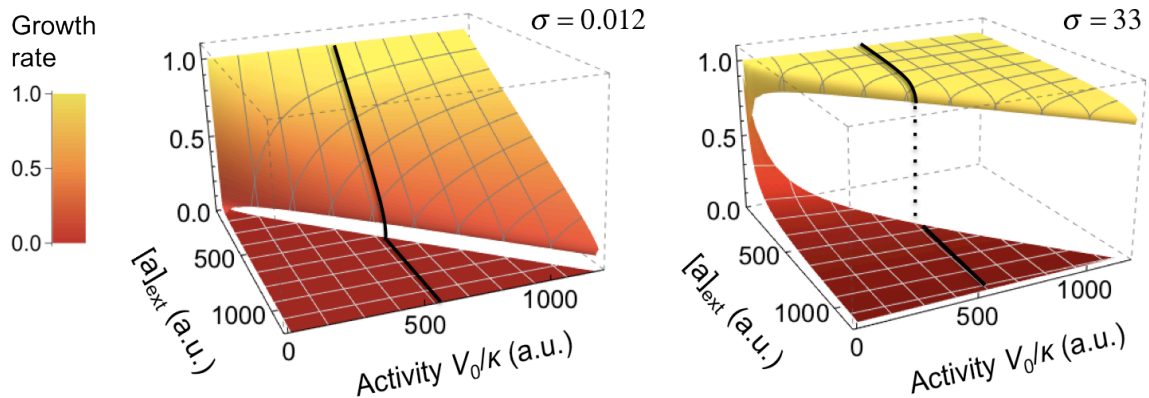


Figure 5.3. Theoretical fitness landscapes for two classes of strains.

Here are two fitness landscapes, each predicting growth rates as a function of resistance enzyme activity and antibiotic concentration $[a]_{\text{ext}}$. Strains in either landscape are described by lines of fixed enzyme activity (constant V_0/k , e.g. the black lines above). If a strain's resistance is provided chiefly by endogenous efflux, then the saturability of the efflux pump for the drug is low, characterized by small $\sigma = I_{50} / K_m$. The landscape on the left (saturability $\sigma = 0.012$) is typical for this low-saturability resistance, in which the Michaelis constant of the efflux pump for the drug is ~ 100 -fold higher than the drug's half-inhibition constant. The landscape on the right ($\sigma = 33$) is an extreme example of a high-saturability resistance system (large σ), in which the Michaelis constant of the efflux enzyme is much lower than the binding affinity of the drug for its target on the ribosome. The striking difference between "plateau heights" (determined by Eq. [5.3]) in the two surfaces demonstrates how fitness landscapes may be distinguished according to their specificities. The Cm-CAT landscape has saturability of order unity, with intermediate plateau height (Figure 5.1). Regions of growth-bistability are omitted from the plots (low-growth branches are truncated at MIC).

5.5 Effects of landscape structure on fixation probabilities

Here I demonstrate (as a simple proof of concept) that the drastically different shapes of these two landscapes can have a significant effect on a microbe's ability to adapt to antibiotic treatment, i.e., to successfully fix a phenotype that provides increased MIC to a population. As parameters I_{50} and K_m ultimately determine the shapes of these landscapes, I will show the way that these parameters can influence the rate of evolution of antibiotic resistance. I am interested here in the appearance of resistant mutants among populations of cells exposed to sub-inhibitory antibiotic concentrations, the prototypical condition for breeding drug-resistant mutants (Baquero & Negri, 1997; Palmer & Kishony, 2013).

5.5.1 A heuristic example

To capture the essential effects of plateau shape on evolution in a heuristic example, I examine two landscapes motivated by the landscapes in Figure 5.3. Specifically, I describe the fixation probability for a mutation occurring in either of the landscapes. As discussed above in section 5.3, sequential mutations in either landscape may be modeled as multiplicative increases in the value of V_0/κ , leading to multiplicative increases in MIC with each mutation. In the following examples, I discuss a single mutant in each landscape whose fixation would result in ~ 2 -fold increase in MIC for the population; the factor of 2 is chosen because it is experimentally observed to arise during *in vitro* evolution as described above.

We first discuss fixation in an extreme example of a mesa-shaped landscape, in which growth rate is a step function taking on a growth rate of either one or zero to

approximate the landscape shown on the right in Figure 5.3. The growth rates of the parent and the single mutant are shown explicitly below, on the right in Figure 5.4. In this landscape a mutant (red) has a higher MIC (vertical line) than the parent strain (black), but cannot increase growth rate any further due to the mesa-like shape of the landscape. Therefore, for any antibiotic concentration in which the parental population can divide and produce mutants (i.e. the interval $(0,1)$ in this example), a mutant does not experience an increase in growth rate, only in an increase in the maximum tolerated MIC.

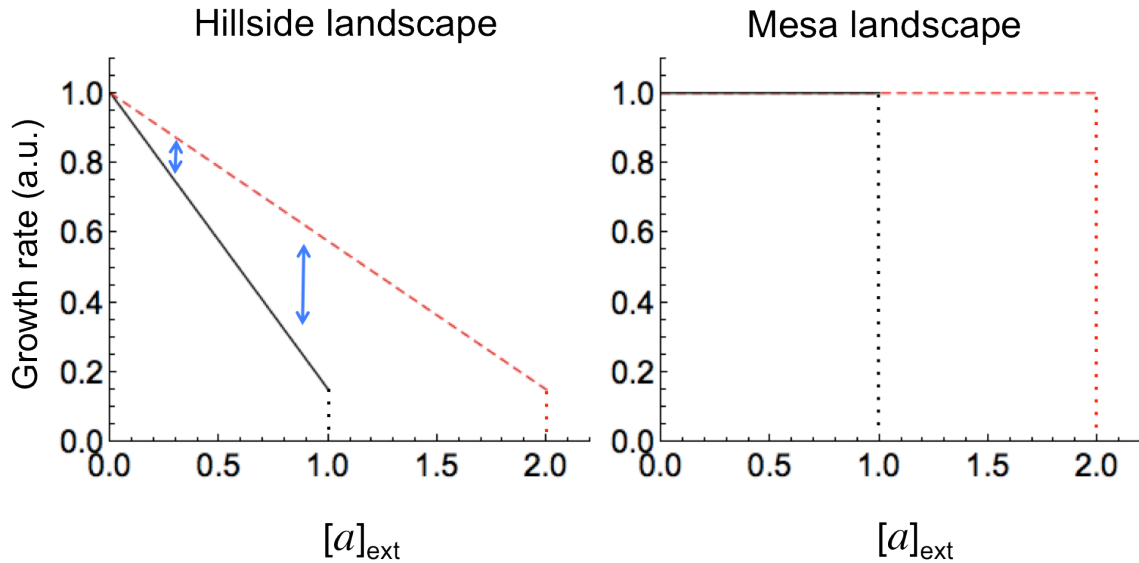


Figure 5.4. Comparison of a single mutation in two landscapes.

Over the course of reproduction, parental strains (growth rates in black) generate a single mutant offspring (growth rates in red). In each landscape, if the mutations become fixed, they will increase the MIC of the population by a factor of two. However, fixation of the mutation is predicted to occur more readily in the hillside landscape because mutants (red) experience a selective advantage that depends on the difference in growth rates between mutant and parent (blue arrows). Mutants in the mesa landscape increase their likelihood of fixation if they can escape competition by invading an uninhabited environment (see (Greulich et al., 2012; Hermesen et al., 2012)). However, mutants in the hillside landscape would be able to do the same in such a scenario.

We assume for the sake of this example that the total number of cells in the population N is fixed rather than growing exponentially (e.g. as in a typical logistic growth-death model with population size at some carrying capacity (Novozhilov, Karev, & Koonin, 2006)), and that mutation rate is low (such that I am only concerned with the fixation of one mutant in the example). If N is constant, then the process of fixation is expected to follow a simple Moran process (Novozhilov et al., 2006). In this example the relative fitness (the growth rate of the mutant divided by the growth rate of the parent, λ_μ / λ_p) is unity, so evolution is neutral, and I have the well-known result that the probability of fixation for a single mutant once it appears is simply

$$P_{\text{fix}} = \frac{1}{N} \quad \left(\lambda_\mu = \lambda_p, \text{MIC}_\mu > \text{MIC}_p \right) \quad [5.5]$$

We turn now to the same process in a hillside-shaped landscape (Figure 5.3 and Figure 5.4, left). A mutation that increases the parameter V_0 / κ by two-fold (the same type of mutation as described for the mesa example above) also increases MIC in proportion (red vertical dashed line), as discussed in section 5.3 and elsewhere. But in contrast to the result in the mesa-shaped landscape, the increase in V_0 / κ is here accompanied by an increased growth rate for the mutant because the landscape is not a step-function. The exact value of the growth rate depends on details (parameter values, drug concentration, etc.) but the important result is that the increase will be non-zero for any antibiotic concentration in which the population can divide and produce mutants (see blue arrows in Figure 5.4). Again using the Moran process as a heuristic for a fixed number of cells N (Novozhilov et al., 2006), the fixation probability is

$$P_{\text{fix}} = \frac{1 - \lambda_p / \lambda_\mu}{1 - (\lambda_p / \lambda_\mu)^N} \quad [5.6]$$

This probability will in general be greater than $1/N$ due to the differences in parental and mutant growth rates, and it is roughly independent of N when population size is large and $\lambda_\mu > \lambda_p$. In the limit that growth rate differences are very small for all drug concentrations, $\lambda_\mu \rightarrow \lambda_p$, the result approaches the neutral probability of the mesa-shaped landscape. This simple analysis shows that fixation probability of a single mutant in the hillside landscape can even become more likely than not, depending on the antibiotic concentration in which the selection occurs. In contrast, the probability of fixation for a single mutant in the mesa landscape will always decrease with N . Therefore, for the same population size N and non-zero antibiotic concentration, mutants arising in a hillside landscape are more likely to fix than those arising in the mesa landscapes, sometimes even dramatically so.

We note here that this difference in fixation probability between the two landscapes will depend on the step-size of mutation (vanishing as step size vanishes), however the size chosen here is in accord with experimental results discussed above. Also, the effect of plateau shape on evolution in an environment of heterogeneous drug concentrations (either in time or space) is more complicated than the picture shown here (and a part of ongoing research). However, a similar trend in which mesa-shaped landscapes suppress fixation of new mutants is expected in general.

By returning from these simplified models to the full fitness landscapes developed in this work, we gain worthwhile insight. The results above suggest that wild-type strains are well poised to fix beneficial mutations, because the hillside landscape characterizes

the fitness of native efflux (see Figure 5.3). Meanwhile, mesa-shaped landscapes, characterized by high saturability ($I_{50} \gg K_m$), make fixation of beneficial mutations (mutations that potentially increase the population's MIC) more difficult. Therefore, all else being equal, a series of mutations throughout the hillside landscape is expected to fix at a faster rate than the equivalent series of mutations along the mesa landscape. However, if two strains from each landscape were to compete in a single environment, the strain from the mesa landscape (high saturability, $I_{50} \gg K_m$) would win due to its selective advantage over the other strain at every drug concentration. Interestingly, the quick fixation of the high-saturability strain over the low saturability strain would essentially halt any future evolution of increased resistance. This scenario raises interesting questions about the evolvability of resistance, and the possibility of designing infection treatment strategies that suppress beneficial mutations, both topics of ongoing research. These issues highlight the significance of the plateau-shaped fitness landscapes that arise naturally in resistance to translation-inhibiting antibiotics.

5.6 Phenotype versus genotype

To conclude, I return to the issue of connecting genotype to phenotype, and discuss possibilities for predicting the quantitative effect of genotype on fitness in antibiotic resistance. With the work outlined in this dissertation, such predictions are already possible.

As a proof of principle I introduce the “RBS calculator,” an online computational tool that predicts mRNA translation-initiation rate from a user's input mRNA sequence

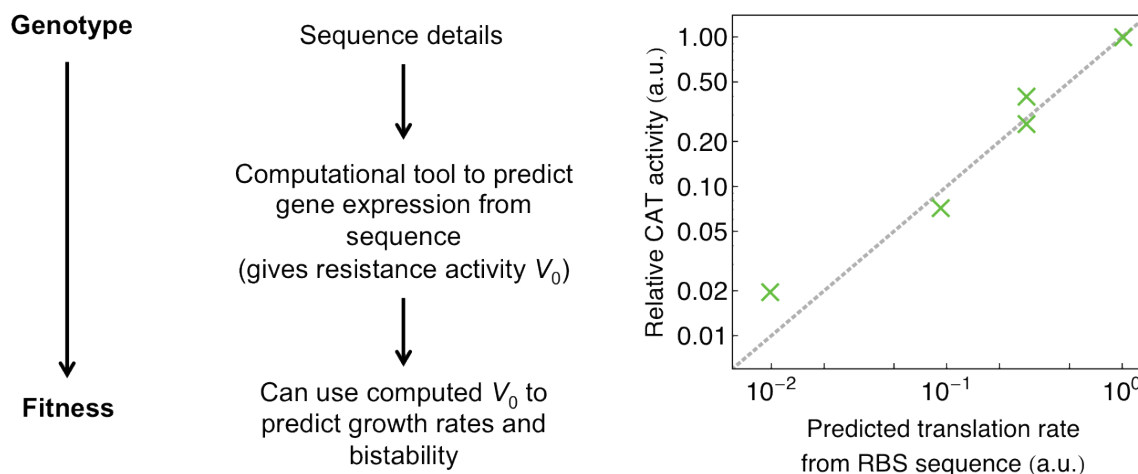


Figure 5.5. Predicting phenotype and fitness from genotype.

Using computational tools and the fitness landscapes developed in this work, it is possible to predict relative fitness of antibiotic resistant strains based on sequence data alone. The “RBS Calculator” is an online tool that predicts translation initiation rates for mRNA transcripts based on their sequences. The plot shows predicted translation initiation rates for *Cm*-resistant strains *Cat1*, and *Cat3-Cat6*. Using these data, one can predict relative MIC or growth rates among the strains as described in the text (by fixing V_0).

(Salis, Mirsky, & Voigt, 2009). Inasmuch as expression of a protein depends linearly upon this initiation rate, then one can use the rates predicted by the tool to estimate the protein amount translated from the mRNA. I input the mRNA sequences of strains *Cat1* and *Cat3-Cat6* into the tool (these are the strains that express CAT from the constitutive pLac-O1 promoter, with mRNA sequences differing only in the bases in the ribosomal binding site region, see Table C.3) to obtain the estimated translation initiation rate on the *cat* mRNA for each strain (relative to the rate predicted for strain *Cat1*). The plot in Figure 5.5 shows that the sequence-based estimates of translation initiation rates predict the measured CAT activities of each strain reasonably well, within about a factor of two.

Then as described above in Figure 4.9, by measuring the MIC of a reference strain

whose mRNA sequence is known (e.g. Cat1), one can fix the parameter V_0/κ for the strain using Eq. [4.13]. Taking the predicted translation initiation rates as *in silico* estimates of CAT activities (as justified by the agreement in Figure 5.5), one could in principle then use the estimates to predict growth behaviors of other strains expressing CAT from the same promoter, but with arbitrary mRNA sequence in the ribosomal binding region upstream from the gene.

This is but one computational tool; recent theoretical and computational work even allows sequence-based predictions of Michaelis constants K_m and k_{cat} (Romero, Krause, & Arnold, 2013). Meanwhile, sequencing technologies continue to grow and have led to an explosion in the volume of DNA sequence data available, a trend that is not likely to reverse (Kodama, Shumway, & Leinonen, 2012). As computational tools like the RBS-calculator become more abundant and better in predictive quality, phenotype-based fitness landscapes like the one developed in this work will be crucial for predicting biological behavior from massive volumes of data—especially in the field of antibiotic resistance, in which sequencing drug-resistant isolates has become the norm (Palmer & Kishony, 2013).

5.7 Acknowledgement

With permission from the coauthors, chapters 2-5 of this dissertation contain work from a manuscript that has been submitted for publication with the working title, “The innate growth bistability of antibiotic resistance,” 2013, Deris, JB; Kim, M; Zhang, Z;

Okano, H; Hermesen, R; Groisman, A; Hwa, T. The dissertation author and Minsu Kim are co-first authors in the submitted work.

Appendix A

Stability analysis

Here I perform a stability analysis of the deterministic model whose steady states are captured by Eqs. [4.1] and [4.4]. Specifically, I address the qualitative question regarding the stability in the coexistence regime where multiple solutions are obtained. By themselves, Eqs. [4.1] and [4.4] provide no information as to whether cells can approach the steady state when antibiotic concentrations are transiently perturbed. However, I can address this kinetic question qualitatively by implementing a quasi-steady-state approximation in which the effect of drug on cell growth (Eq. [4.4]) and the growth-rate dependence of enzyme levels (Eq. [3.25]) remain satisfied as internal antibiotic concentration changes. In this limit I write the rate of change of the internal concentration a_i as

$$\frac{da_i}{dt} = \kappa \cdot \left(a_e - a_i \cdot \left(1 + \rho \frac{1}{1 + a_i} \frac{1}{1 + \sigma \cdot a_i} \right) \right) \equiv f(a_i) \quad [\text{A.1}]$$

This form is dictated by the effects of drug influx and efflux (or modification, degradation) on the internal drug concentration. Eq. [4.1] gives the steady state condition

of Eq. [A.1], i.e., $f(a_i) = 0$.

We perform linear stability analysis with respect to changes in concentration by perturbing about the steady state solution $a_{i,s}$, defined by $f(a_{i,s}) = 0$. The expected response to small perturbations $\delta a_i = a_i - a_{i,s}$ is $\delta f = c \cdot \delta a_i$, with

$$c \equiv \left. \frac{\partial f}{\partial a_i} \right|_{f=0} = -\kappa \cdot \left. \frac{\partial}{\partial a_i} a_e(a_i; \rho, \sigma) \right|_{a_i=a_{i,s}} \quad [\text{A.2}]$$

and using Eq. [4.1] for the dependence of $a_{i,s}$ on a_e . Stability requires $c < 0$ such that the system returns to the steady state for small perturbations away from the stationary solution. The sign of c can be easily determined graphically, by inspecting the form of $a_e(a_i)$ as depicted in Figure 4.1: We see that $\partial a_e / \partial a_i < 0$ only in the intermediate (red) segment in between the two extrema. Thus, $c > 0$ and the solution $a_{i,s}$ in the intermediate range between a_i^* and $a_i^\dagger$ is unstable, while everywhere else the solution is stable. This geometric statement can be readily proved algebraically for any values of the parameters for this system.

Appendix B

Independent estimates of the cell's permeability to chloramphenicol

To determine whether the prediction of $\kappa \sim 1.5 \text{ s}^{-1}$ for chloramphenicol (based on MIC and CAT activity of Cat1, Table C.2) is reasonable, I estimate here the specific permeability of *E. coli*'s inner- and outer-membranes (IM, OM) to Cm using primarily the method outlined by Thanassi *et al.* (Thanassi et al., 1995), which is based on Cm hydrophobicity, charge, etc. Then I compose an effective permeability constant from the specific permeabilities with Eq. [3.12].

B.1 Inner membrane permeability

Collander (Collander, 1954) demonstrated that a molecule's specific permeability π through a typical lipid bilayer membrane (similar in composition to the inner membrane in *E. coli*) is related to the molecular weight (MW) and the partition coefficient c of the molecule in ether-water such that $\log(c^{1.3}) \sim \log(\pi \cdot MW^{1.5})$ at 20°C. The apparent partition coefficient of Cm in ether-water for $2.0 \leq \text{pH} \leq 9.0$ is given (Bartz, 1948) at

25°C as $c_{25}=3$. However, to use Collander's relation and obtain permeability, I must correct for the temperature dependence of partition coefficients (Stein, 1967) $\pi(T) \propto e^{-E_a/RT}$. I take Pramer's (Pramer, 1959) value of activation energy $E_a \sim 10^4$ cal/mol, based on the diffusion of Cm into *Nitella* cytoplasm; this number is comparable to values for other antibiotics passing solely through lipid bilayer (Stein, 1967; Thanassi et al., 1995). This correction gives $c_{20} \approx 2.2$. Collander's data then suggest that for molecules with MW and partition coefficient similar to those of Cm, the permeability falls in the range $10^{-5} \leq \pi \leq 10^{-4}$ cm/s. To obtain the specific permeability at 37° C, I again correct for temperature to obtain

$$2.6 \times 10^{-5} \lesssim \pi_{I.M.} \lesssim 2.6 \times 10^{-4} \text{ cm/s} . \quad [\text{B.3}]$$

B.2 Outer membrane permeability

Estimating outer membrane permeability is less direct and I must appeal to permeabilities of other compounds. Chloramphenicol diffusion through OM porins is much faster than through lipopolysaccharide, and loss of porin production significantly decreases the ability of Cm to inhibit growth (Mortimer & Piddock, 1993; Nikaido, 2003). Cm diffuses through PhoE, OmpC, and OmpF porins; molecular weight, charge, size, and hydrophobicity have all been found to influence the permeability of antibiotics through these OM porins (Mortimer & Piddock, 1993; Nikaido, 2003). For the lack of direct data on chloramphenicol permeability, I use the permeabilities of two drugs with similar size and total charge (tetracycline and cephalexin) through the OmpF porin as reference points. By combining the data due to Nikaido *et al.* (Nikaido, Rosenberg, &

Foulds, 1983; Yoshimura & Nikaido, 1985), I obtain absolute permeability rates of various compounds through porin-containing membrane as a function of hydrophobicity, with hydrophobicity given by the logarithm of the partition coefficient in n-octanol. The partition coefficient of Cm in n-octanol is reported (Wu, Clift, Fry, & Henry, 1996) as $c_{n-oct} = 12$. Electrically neutral compounds of similar size and hydrophobicity as Cm (like cephalalexin), have permeability through OmpF of $\sim 4 \times 10^{-4}$ cm/s (Yoshimura & Nikaido, 1985). Thanassi *et al.* (Thanassi et al., 1995) give a permeability of the bulkier tetracycline through these porins $\sim 10^{-5}$ cm/s. I therefore estimate $10^{-5} \leq \pi_{O.M.} \leq 10^{-4}$ cm/s for chloramphenicol.

B.3 Comparing model predictions with estimated permeability

Then using Eq. [3.12] I combine these specific inner- and outer-membrane permeabilities and cytoplasmic volume Ω_{cyt} to compute the effective permeability for both membranes together

$$0.37 \lesssim \kappa \lesssim 7.5 \text{ s}^{-1} \quad [\text{B.4}]$$

with $A_i = 105 \text{ cm}^2/\text{mg}$, $A_o = 132 \text{ cm}^2/\text{mg}$, and $\Omega_{cyt} = 0.0024 \text{ cm}^3/\text{mg}$ (all mass units are dry weight of cells) (Thanassi et al., 1995), although I use these parameter values only as an order of magnitude estimate, and in particular the *in vitro* upper bound may be somewhat higher. Table C.2 shows that the predicted value of permeability $\kappa_{Cat1} \sim 1.5 \text{ s}^{-1}$ for strain Cat1 is within this estimated range. Similarly, with V_0 fixed for all CAT-expressing strains (Figure 4.7, bottom), the parameter fitting of all CAT-expressing strains for which

growth rates were measured (colored lines, Figure 4.10), gives an average permeability for all strains $\kappa_{\text{all}} \sim 1.5 \pm 0.14 \text{ s}^{-1}$ (s.d.).

Because the estimated range for κ is based solely on physical properties like the hydrophobicity, and on permeability of cells away from steady-state growth, I offer the following caveat: In the simple model (Eq. [3.30]), I did not explicitly include the effects of low-affinity native efflux and porin regulation; therefore the permeability parameter κ is an effective one including such effects. (For similar reasons, I acknowledged near Eq. [3.4] that the fit value of the half-inhibition constant I_{50} might actually represent an effective $\tilde{I}_{50}$). Therefore, I expect this κ , representing an effective permeability of the cell at steady state (from Table C.2), to be lower than a value estimated on physical properties alone.

Appendix C

Tables

Table C.1. Bacterial strains

All strains used in the study. Experiment details of construction for each strain are given in experimental methods section of each relevant chapter.

Strain	Genotype	Background	Comments	AKA
EQ4 “wild type”	$\Delta lacIZY$	MG1655	<i>E. coli</i> K-12 MG1655 with the native <i>lac</i> operon removed	—
EQ4m	$\Delta motA$	EQ4	immotile wild type strain	EQ79
Lac1	$P_{Llac-O1}-lacZ$	EQ4	constitutive expression of <i>lacZ</i> at the <i>lac</i> locus, driven by the synthetic $P_{Llac-O1}$ promoter	EQ5
Lac2	$P_{Ltet-O1}-lacZ$	MG1655 $\Delta lacI$, $\Delta galK$, $\Delta rhyB$	constitutive expression of <i>lacZ</i> at the <i>lac</i> locus, driven by the synthetic $P_{Ltet-O1}$ promoter	EQ37 from ref. (Scott et al., 2010)
Ta1	$Km^r:rrnBT: P_{Llac-O1}-tetA$	EQ4	constitutive expression of <i>tetA(B)</i> from transposon Tn10 at the <i>ycaD</i> locus	EQ93
Cat1	$rrnBT:$ $P_{Llac-O1}-RBS1-cat$	EQ4	constitutive expression of <i>cat</i> by the synthetic $P_{Llac-O1}$ promoter at the <i>ycaD</i> locus; employs “RBS1” as the Shine-Dalgarno (SD) sequence*	EQ75
Cat2	$Km^r:rrnBT: P_{Ltet-O1}-cat$	EQ4	constitutive expression of <i>cat</i> by the synthetic $P_{Ltet-O1}$ promoter at the <i>ycaD</i> locus; employs RBS1 as SD sequence	EQ92
Cat3	$rrnBT:$ $P_{Llac-O1}-RBS3-cat$	EQ4	as Cat1, but employs “RBS3” as the SD sequence	EQ94
Cat4	$rrnBT:P_{Llac-O1}$ $P_{Llac-O1}-RBS4-cat$	EQ4	as Cat1, but employs “RBS4” as the SD sequence	EQ95
Cat5	$rrnBT:$ $P_{Llac-O1}-RBS5-cat$	EQ4	as Cat1, but employs “RBS5” as the SD sequence	EQ96
Cat6	$rrnBT:$ $P_{Llac-O1}-RBS6-cat$	EQ4	as Cat1, but employs “RBS6” as the SD sequence	EQ98
Cat1m	$\Delta motA$, $rrnBT:$ $P_{Llac-O1}-RBS1-cat$	EQ4m	as Cat1, but immotile	EQ107
GCat1m	$rrnBT:$ $P_{Llac-O1}-RBS1-cat$, $\Delta motA$, $Km^r:$ $rrnBT:P_{Ltet-O1}-gfp$	EQ4m	as Cat1m, but also harboring constitutive expression of <i>gfp</i> at the <i>intC</i> locus	EQ108

* See Table C.3 for ribosomal binding site “RBS” sequences

Table C.2. Biochemical parameters related to strain Cat1

Parameter (units)	Description [§]	From literature, or measured	Fit from data	Predicted [†]
λ_0 (hr ⁻¹)	growth rate in the absence of Cm	0.62 ± 0.03^a	—	—
K_D (μM)	Cm-ribosome dissociation constant	$0.6 - 2.7^b$	—	1.3 ± 0.12^k
K_m (μM)	Michaelis constant for CAT enzyme	12^c	—	—
k_{cat} (s ⁻¹)	turnover number for CAT enzyme	600^c	—	—
κ (s ⁻¹)	membrane permeability to Cm	$0.37 - 7.5^d$	—	1.5^l
V_0 (μM/OD ₆₀₀ /s)	CAT activity in the absence of Cm	3.7 ± 0.3^e	—	—
I_{50} (μM)	half-inhibition conc.	—	5.5 ± 0.5^h	$2.8 - 12^k$
V_0 / κ (mM)	CAT activity relative to permeability	—	5.8 ± 0.5^i 5.78 ± 0.08^j	$0.90 - 18^m$
MIC (μM)	min. inhibitory conc.	$950 \pm 50^{f,g}$	—	950 ± 10^n

[§] All *in vivo* quantities refer to Cat1 cells growing exponentially in minimal glucose medium with Cm unless otherwise indicated.

[†]These values are predicted using other parameter values listed in the two left columns; they are provided to illustrate consistency between model results and literature.

^a. Growth rate of strain Cat1 in glucose minimal media in absence of antibiotics, fit from slope of log-OD versus time with standard deviation from four replicates.

^b. The range of dissociation constant values reported for Cm-ribosome binding (Contreras & Vázquez, 1977; Harvey & Koch, 1980).

^c. Michaelis constant and the turnover number for CAT enzyme (Ellis et al., 1995). Error for the K_m value is described as “less than 10%” in the reference.

^d. Membrane permeability for Cm was estimated using the methods of Nikaido (Thanassi et al., 1995). Briefly, permeability of Cm through cytoplasmic membrane is calculated based on Cm hydrophobicity and molecular weight (Bartz, 1948; Collander, 1954). Permeability through *E. coli* outer membrane is calculated using available data for permeabilities of antibiotics of similar hydrophobicity, molecular weight, size and charge (Nikaido et al., 1983; Nikaido & Thanassi, 1993; Yoshimura & Nikaido, 1985). Based on simple flux balance across each membrane, an effective permeability κ is

defined in terms of cytoplasmic and outer membrane permeabilities using reported dimensions of cell volume and membrane surface area (Thanassi et al., 1995) (see section 3.3.3.2).

- e. V_0 , the CAT activity of exponentially growing Cat1 cells in Cm-free medium, is quantified using a spectrophotometric assay (Shaw, 1975) (see experimental details in section 4.7.3). I provide the estimated *in vivo* activity here as acetylation rate per cytoplasmic volume by the following conversion. I divide the molar rate of Cm acetylated/OD₆₀₀ by a factor (Bipatnath, Dennis, & Bremer, 1998) of 1.6 to convert our measurements from per-OD₆₀₀ to per-OD₄₆₀, and then divide by cell volume per mass (Churchward, Estiva, & Bremer, 1981) of $0.34 \pm 0.04 \mu\text{L cells}/(\text{mL culture OD}_{460})$ to obtain the rate relative to equivalent cell volume in the reaction. Cell volume per mass changes little across strains or growth conditions in *E. coli* (Begg & Donachie, 1978; Bremer & Dennis, 1987; Churchward et al., 1981). I finally divide this quantity by 0.8 to take into account that cytoplasm comprises approximately 80% of total cell volume in *E. coli* K-12 (Cayley, Lewis, Guttman, & Record, 1991; Stock, Rauch, & Roseman, 1977) to obtain the result in desired units: $V_0 \approx 8.5 \pm 0.8 \text{ mM/s}$.
- f. MIC from the abrupt growth rate drop in batch cultures. See, for example, growth curves in Figure 3.6.
- g. MIC_{plate} from LB plating, see Figure 2.3.
- h. The value of half-inhibitory concentration obtained from the fit of the growth data of wild type cells in sub-inhibitory concentrations of Cm (Figure 3.3) to equation [3.23], assuming $[a]_{\text{int}} \sim [a]_{\text{ext}}$. I performed a simple weighted least squares fit using weights $w = (\text{s.e.})^{-2}$ for each data point, with s.e. given by the standard error of doubling time for each determination (error bars in Figure 3.3). Uncertainty in the fit parameter I_{50} is a function of the estimated variance and is reported as standard error of the regressed parameter, with estimated variance $\sigma^2 = \sum_i w_i \cdot (\text{fit residual}_i)^2 / (\text{D.F.})$.
- i. Parameter V_0/κ is obtained by solving equation [4.13] for the empirical (batch culture) MIC obtained for strain Cat1, with the value MIC=950 μM taken from (f,g), the value of $I_{50} = 5.5 \mu\text{M}$ taken from (h), and K_m from (c). Error represents uncertainties propagated from MIC, I_{50} , and K_m .
- j. This is an alternative estimate obtained by fitting the growth rate data of Figure 3.7 with an equation for $\lambda([a]_{\text{ext}})$ obtained from equations [3.30]-[3.21], with V_0/κ as the only free parameter (as described in Figure 4.10). To fit the data independently of MIC, data from non-growing cultures were excluded from fit and I used only the data for which growth rates were non-zero. This value obtained by fitting growth rates is similar to the value predicted by MIC in (f).
- k. The half-inhibitory concentration is expected to be proportional to the Cm-ribosome dissociation constant as discussed in section 3.4.2 ($I_{50} = K_D \lambda_{\text{max}}/\lambda_0$, equation [3.22]), independent of a strain's CAT activity. To provide the value of K_D predicted by the model and growth data, I solve this equation for K_D using the fit value of $I_{50} = 5.5 \mu\text{M}$ from (h), $\lambda_0 = 0.67 \text{ hr}^{-1}$ for WT strain EQ4 from (a), and $\lambda_{\text{max}} = 2.85 \text{ hr}^{-1}$ from Scott *et*

aI^9 . Conversely, I also calculate an estimate for I_{50} using equation [3.22] and the K_D range from the literature given in (b).

- ^l. We obtain this estimate of $\kappa = 1.5 \text{ s}^{-1}$ from the $V_0 / \kappa = 5.8 \text{ mM}$ obtained by MIC (f), and the in vitro value for V_0 reported in (e). In Figure 4.9, I obtain the V_0 / κ values for strains Cat2-Cat6 by assuming all strains have this same permeability to Cm. With this assumption, V_0 / κ for all strains are determined simply by the relative CAT activities displayed in Figure 3.7 (grey bars).
- ^m. This estimate of the key parameter of the model uses the parameter values V_0 and κ given in (e) and (d) respectively.
- ⁿ. MIC calculated using the value of V_0 / κ obtained from fitting the model to growth rates (j) and equation [4.13], with the value of $I_{50} = 5.5 \text{ }\mu\text{M}$ taken from (h), and K_m from (c). Error propagated from standard error of fit in (j) and uncertainties of I_{50} and K_m .

Table C.3. RBS substitutions in CAT strains

Modifications of the RBS region in constitutive promoters. The -35 (TTGACA) and -10 (GATACT) elements are underlined and the transcriptional start site (+1; indicated a bolded capital "A") and the first codon (ATG) are bolded. RBS is highlighted in cyan, nucleotide substitutions in yellow, and nucleotide insertion in magenta. RBS1=original RBS; RBS3=4 base insertion; RBS4=1-base substitution; RBS5= 1-bp substitution plus 4-base insertion; and RBS6=2-bp substitution plus 4-base insertion.

RBS ID	Strain	P _{Lac-O1} and RBS
RBS1	Cat1	CTCGAGAATTGTGAGCGGATAACAATTGACATTGTGAGCGGATAACAAGATACT GAGCAC A TCAGCAGGACGCACTGACCGAATTCATTAAAGAGGAGAAAGGTACCA A TG
RBS3	Cat3	CTCGAGAATTGTGAGCGGATAACAATTGACATTGTGAGCGGATAACAAGATACT GAGCAC A TCAGCAGGACGCACTGACCGAATTCATTAAAGAGGAGTCCTAAAGGT ACCATG
RBS4	Cat4	CTCGAGAATTGTGAGCGGATAACAATTGACATTGTGAGCGGATAACAAGATACT GAGCAC A TCAGCAGGACGCACTGACCGAATTCATTAAAGAGGACAAAGGTACCA A TG
RBS5	Cat5	CTCGAGAATTGTGAGCGGATAACAATTGACATTGTGAGCGGATAACAAGATACT GAGCAC A TCAGCAGGACGCACTGACCGAATTCATTAAAGAGGACTCCTAAAGGT ACCATG
RBS6	Cat6	CTCGAGAATTGTGAGCGGATAACAATTGACATTGTGAGCGGATAACAAGATACT GAGCAC A TCAGCAGGACGCACTGACCGAATTCATTAAAGAGGCCCTCCTAAAGGT ACCATG
		P _{Ltet-O1} and RBS
RBS1	Cat2	CTCGAGTCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACT GAGCAC A TCAGCAGGACGCACTGACCGAATTCATTAAAGAGGAGAAAGGTACCA A TG

Table C.4. Primers used in this study

Primer	Sequence (5' to 3')	Use
Cat-Kpn-F	ATAGGTACCATGGAGAAAAAATCAC TGGATATAC	Cloning <i>cat</i> into pKDT_ P _{Llac-O1} or pKDT_ P _{Ltet}
Cat-Bam-R	ATTGGATCCTTACGCCCCGCCCTGCCA CTC	Cloning <i>cat</i> into pKDT_ P _{Llac-O1} or pKDT_ P _{Ltet}
tetA-Kpn-F	AATGGTACCATGAATAGTTCGACAAA GATCGCATTG	Cloning <i>tetA</i> into pKDT_ P _{Llac-O1}
tetA-Bam-R	TATGGATCCTAAGCACTTGTCTCCTGT TTACTC	Cloning <i>tetA</i> into pKDT_ P _{Llac-O1}
Plac-RBS1	CATTGGGATATATCAACGGTGGTATAT CCAGTGATTTTTTCTCCATGGTACCTT TAGGACTCCTCTTTAATGAATTC	RBS region mutation in P _{Llac-O1}
Plac-RBS3	CATTGGGATATATCAACGGTGGTATAT CCAGTGATTTTTTCTCCATGGTACCTT TGTCTCTTTAATGAATTC	RBS region mutation in P _{Llac-O1}
Plac-RBS4	CATTGGGATATATCAACGGTGGTATAT CCAGTGATTTTTTCTCCATGGTACCTT TAGGAGTCCTCTTTAATGAATTC	RBS region mutation in P _{Llac-O1}
Plac-RBS5	CATTGGGATATATCAACGGTGGTATAT CCAGTGATTTTTTCTCCATGGTACCTT TGGCCTCTTTAATGAATTC	RBS region mutation in P _{Llac-O1}
Plac-RBS6	CATTGGGATATATCAACGGTGGTATAT CCAGTGATTTTTTCTCCATGGTACCTT TAGGAGGCCTCTTTAATGAATTC	RBS region mutation in P _{Llac-O1}
PlaclacZ-P1	GCATTTACGTTGACACCATCGAATGGC GCAAAACCTTTCGCGGTATGTGTAGGC TGGAGCTGCTTC	Chromosomal substitution of P _{Llac-O1} to <i>lacI</i> and <i>PlacZYA</i>
PlaclacZ-P2	CGTTGTAAAACGACGGCCAGTGAATCC GTAATCATGGTCATAGCTGTTTTCTCCT CTTTAATGAATTCGG	Chromosomal substitution of P _{Llac-O1} to <i>lacI</i> and <i>PlacZYA</i>
Lac-ver-R	CAACTGTTGGGAAGGGCGATCGGTG	Verification of chromosomal P _{Llac-O1}
Cat-P1	AGACGCGATGCATTGCTCTGAAAGCAT AGACGGGAAATATGAGTTTGCTGTGTA GGCTGGAGCTGCTTC	Chromosomal integration of P _{Llac-O1} - <i>cat</i> or P _{Ltet} - <i>cat</i> to <i>ycaD</i> locus
Cat-P2	GGTGAAAATACGCGATATCCCAGCGG CGGTATTATCGATTATATTACATGAG AATTAATCCGGGGATCC	Chromosomal integration of P _{Llac-O1} - <i>cat</i> or P _{Ltet} - <i>cat</i> to <i>ycaD</i> locus
ycaD-ver-R	GCCAGAGTCAACAAAAGCAGGC	Verification of chromosomal P _{Llac-O1} - <i>cat</i> or P _{Ltet} - <i>cat</i>

Table C.5. Biochemical parameters related to the TetA system

Parameters for Tc (top) and Mn (bottom).

Parameter (units)	Description	From literature, or measured	Fixed by MIC	Fit in model*	Predicted
λ_0 (hr ⁻¹)	growth rate in absence of antibiotic	0.68 ^a	—	—	—
K_d^{Tc} (μM)	Tc-ribosome binding dissociation constant	1 ^b	—	—	—
K_m^{Tc} (μM)	Michaelis constant for TetA pump and Tc	10 ^c	—	—	—
I_{50}^{Tc} (μM)	growth half-inhibition constant for Tc	—	—	3.6 ± 0.4 ^d	4.2 ^e
V_0 / κ_{Tc} (mM)	TetA-Tc activity relative to Tc permeability in strain Ta1	—	4.20 ± 0.30 ^f	4.41 ± 0.12 ^d 4.43 ± 0.08 ^g	—
K_d^{Mn} (μM)	Mn-ribosome binding dissociation constant	0.2 ^h	—	—	—
K_m^{Mn} (μM)	Michaelis constant for TetA pump and Mn	2 ⁱ	—	—	—
I_{50}^{Mn} (μM)	Growth half-inhibition constant for Mn	—	—	0.89 ± 0.1 ^j	0.84 ^k
V_0 / κ_{Mn} (μM)	TetA-Mn activity relative to Mn permeability in strain Ta1	—	368 ± 16 ^l	334 ± 22 ^j 324 ± 14 ^m	—

§ All *in vivo* quantities refer to Cat1 cells growing exponentially in minimal glucose medium with indicated antibiotic unless otherwise noted.

*“Fit in model” parameters show that fitting the model to empirical growth rates gives similar results to those predicted by MIC alone. The fit values for I_{50} also illustrate consistency with values calculated from *in vitro* data.

[†]These values are predicted using other parameter values listed in the far left column; they are provided to illustrate consistency between model results and predictions based on literature.

- a. Growth rate of strain Ta1 determined in glucose minimal media batch culture in absence of antibiotics
- b. The dissociation constant for Tc-ribosome binding to its single high-affinity site (Buck & Cooperman, 1990; Epe & Woolley, 1984).
- c. Michaelis constant for TetA efflux of tetracycline (Thanassi et al., 1995).
- d. Here, the half-inhibitory concentration I_{50} and TetA activity V_0/κ were obtained by simultaneously fitting the model (equations [3.30]-[3.21]) to growth rate data of strain Ta1 growing in Tc, with K_m taken from (c), and λ_0 from (b). Errors are standard error of fit, and fitting procedures are identical to those used for Cm-CAT system described in Figure 4.10, except that I fit both V_0/κ and I_{50} simultaneously.
- e. The half-inhibitory concentration is expected to be proportional to the Tc-ribosome dissociation constant as discussed in the 3.4.2 ($I_{50} = K_D \cdot \lambda_{\max} / \lambda_0$, equation [3.22]), independent of a strain's efflux activity, and is calculated here with K_D given in (b), $\lambda_0 = 0.68 \text{ hr}^{-1}$ of strain Ta1 given in (a), and $\lambda_{\max} = 2.85 \text{ hr}^{-1}$.
- f. This parameter is obtained by solving equation [4.13] for V_0/κ with the empirical MIC obtained for strain Ta1 in Tc (MIC = $650 \pm 50 \text{ } \mu\text{M}$, Figure 3.8A), the value of I_{50}^{Tc} taken from (e), and K_m from (c). Error represents uncertainties propagated from MIC.
- g. Here I fit V_0/κ as the only free parameter in the model to growth rate data of strain Ta1 growing in Tc with the value of I_{50}^{Tc} taken from (e), and K_m taken from (c). Errors are standard error of fit, and fitting procedures are identical to those used for Cm-CAT system described in Figure 4.10.
- h. The dissociation constant for Mn-ribosome binding is obtained from the relative binding-affinities of minocycline and tetracycline for 30S-ribosome, given by competitive binding assays between [^3H]-tetracycline and each compound (Table C.3 in Olson *et al.* (Olson et al., 2006)). EC_{50} for each compound gives the concentration at which [^3H]-tetracycline binding is reduced by 50% due to competitive binding. Then I use $K_D^{\text{Mn}} = \frac{\text{EC}_{50}^{\text{Mn}}}{\text{EC}_{50}^{\text{Tc}}} \cdot K_D^{\text{Tc}} = 0.2 K_D^{\text{Tc}}$, with K_D^{Tc} taken from (b).
- i. Michaelis constant for TetA efflux of minocycline is taken as the concentration of minocycline required to inhibit TetA-mediated influx of [^3H]-tetracycline into everted membrane vesicles by 50%, reported elsewhere (Bergeron et al., 1996; Nelson et al., 1993).
- j. The half-inhibitory concentration I_{50} and TetA activity V_0/κ for Mn are obtained as in note (e), except using growth rate data from growth in Mn, with K_m taken from (i).

- ^k. The half-inhibitory concentration is expected to be proportional to the Mn-ribosome dissociation constant as discussed in section 3.4.2 ($I_{50} = K_D \cdot \lambda_{max} / \lambda_0$), independent of a strain's efflux activity, and is calculated here with K_D given in (h).
- ^l. This parameter is obtained by solving equation [4.13] for V_0 / κ with the empirical MIC obtained for strain Ta1 in Mn (MIC $\approx 58 \pm 3$ μ M, see Figure 3.8B), the value of I_{50}^{Mn} taken from (k), and K_m from (i). Error represents uncertainties propagated from MIC.
- ^m. This V_0 / κ was obtained by fitting the model as in note (g), except using growth rate data from growth in Mn with I_{50}^{Mn} taken from (k), and K_m from (i).

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