Biological costs and benefits of antibiotic resistance

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Biological costs and benefits of antibiotic resistance

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Summary

Antibiotic resistance is a large and growing concern for public health. Resistance frequencies to virtually all antibiotics are on the rise and multi-resistant infections are increasingly common. The persistence and spread of antibiotic resistance is driven by their biological costs and benefits. In this thesis I look at the costs and benefits of antibiotic resistance from several different angles.

In **CHAPTER 1** we look at the high variability of costs of resistance depending on the environment they were measured in. We suggest that by differentiating between ‘trait effects’, that is direct effects of the resistance mechanisms on individual traits such as growth rate and yield, and ‘selective effects’, that is effects on genotype frequency over time, we can better understand this variability. A thorough understanding of this variability and how it comes about can potentially help to identify interventions that maximize the costs of resistance.

**CHAPTER 2** presents a detailed investigation of the costs and benefits of the tetracycline efflux pump encoded by \textit{tetA} and how bacteria use gene regulation to optimize these costs and benefits. This efflux pump confers resistance by efficiently removing the antibiotic from the cell and its expression is regulated by a specific repressor, such that it is only produced in the presence of the drug. We confirm that expression of \textit{tetA} is costly and show that the benefits of \textit{tetA} depend on both the expression level of \textit{tetA} and the concentration of
tetracycline.

The following chapter, Chapter 3, takes a closer look at a potential problem of the resistance mechanisms discussed in the previous chapter. *tetA* is only expressed after tetracycline enters the cell where it acts on the ribosome to inhibit protein synthesis. This means that production of the efflux pump could be hindered by the antibiotic, making it difficult for cells to become phenotypically resistant, especially when the appearance of the drug is sudden. We show that there are in fact many genetically resistant cells that fail to divide after sudden exposure to tetracycline while the others continue to divide without a significant lag phase.

In Chapter 4 we show that the evolutionary history can have effects on the competitive costs of newly acquired resistance genes. We experimentally evolved resistant and sensitive strains in the absence of antibiotics and show that costs of additional resistance genes are generally lower in strains that have evolved in the absence of drugs.

Biological costs and benefits of resistance are main drivers of the evolution, persistence and spread of resistance. Understanding how these costs and benefits are modulated by bacteria (Chapter 2 and Chapter 3), how they depend on the environment (Chapter 1) and on evolutionary history (Chapter 4) can help us to manage antibiotic resistance and thus extend the usefulness of antimicrobial therapy.
Zusammenfassung


In KAPITEL 2 behandeln wir, wie durch die Regulation des Gens tetA die Kosten und Nutzen von Tetracyclineresistenz optimiert werden. tetA kodiert für die Tetracycline Effluxpumpe TetA. Diese Effluxpumpe verleiht Resistenz gegen Tetracyclin, indem das Antibiotika aus der Zelle herausgepumpt wird, und ist durch einen Repressor derart reguliert, dass die Pumpe nur produziert wird, wenn das Anti-
ZUSAMMENFASSUNG


In KAPITEL 4 zeigen wir, dass die evolutionäre Vorgeschichte von Bakterien einen Einfluss auf die kompetitiven Kosten von zusätzlichem Resistenzgenen hat. Wir haben sowohl resistente als auch sensitive Bakterien ohne Antibiotika evolviert und haben festgestellt, dass die Kosten von zusätzlichen Resistenzgenen in den evolvierten Stämmen im allgemeinen kleiner sind als bei ihren nicht evolvierten Vorfahren. Die hauptsächlichen Faktoren, die die Evolution, die Verbreitung und die Stabilität von Resistenzen beeinflussen, sind die biologische Kosten und Nutzen von Resistenzgenen. Ein besseres Verständnis dafür, wie die Kosten und Nutzen von der Umwelt abhängen (KAPITEL 1), wie sie von Bakterien moduliert werden (KAPITEL 2 und KAPITEL 3) und wie die evolutionäre Vorgeschichte sie beeinflusst (KAPITEL 4), kann uns helfen, Antibiotikaresistenz besser zu kontrollieren, und so die Lebensdauer von Antibiotika verlängern.
Antibiotic resistance is a growing concern to global public health (Levy and Marshall 2004; Bergstrom and Feldgarden 2008). This is by no means a new problem. Resistances to early antibacterials have been popping up in hospitals soon after their introduction (Barber and Rozwadowska-Dowzenko 1948) and the first problematic multi-drug resistant enteric bacteria were described soon after that (Watanabe 1963). With the increasing use, and misuse, of antibiotics since their introduction, has come an increase in the prevalence of pathogens resistant to one or more antibiotics (Laxminarayan et al. 2013). In Switzerland, for example, the prevalence of multi-drug resistant Neisseria gonorrhoeae (defined as resistant to three or more antibiotics) has increased by a factor of ten between 1998 and 2012 (Endimiani et al. 2014) and trends in other countries are no less worrying. At the same time as rates of resistance are steadily increasing the supply of new antibiotics that previously managed to displace concerns about the loss of effectivity of existing drugs has decreased dramatically (CDC 2013). In light of these developments there is now real concern that the rise in the prevalence of resistance and the lack of new drugs could mean a return to a “preantibiotic era for many types of infections” (Spellberg et al. 2008).

One main effort to combat this problem is the conservation of existing drugs (Laxminarayan 2014). This involves prudent use of antibiotics but also the management of resistance. With most antibiotics being
natural products (Allen et al., 2010), resistance genes are already present at least in the producers and are easily shared with other bacteria, including potential human pathogens (Davies and Davies, 2010). Various selective forces act on resistance genes in natural environments and human use of antibiotics in medicine and agriculture have added additional positive selection pressure leading to the increased prevalence of resistance genes in almost all studied environments (Allen et al., 2010). Once prevalent in the environment, antibiotic resistance is surprisingly persistent in bacterial populations even tough it often incurs a biological costs in the absence of antibiotics. Biological costs of resistance can be defined as negative effects on traits other than drug resistance, which may be present both in the presence and in the absence of drugs.

How does a resistance gene affect a certain trait such as growth rate or yield? Any process not directly needed for growth in the current environment can have costs in terms of protein material cost (Bragg and Wagner, 2009) and energy costs (Wagner, 2005). This metabolic burden alters the biochemistry and physiology of the bacterial cell (Glick, 1995) and can lead to the observed trait effects. There are also other processes related to resistance genes that can alter trait effects. Expressing any additional genes will divert ribosomes away from other genes necessary for growth (Scott et al., 2010). The availability of free ribosomes limits protein synthesis (Vind et al., 1993) and can thus influence growth rate. Another source for costs is molecular crowding (Klumpp et al., 2009). Slow diffusion of tRNA complexes in the crowded cytoplasm puts a physical limit on the speed of translation and additional proteins will increase this problem. The activity of the resistance gene could also contribute to a reduction in a trait effect. This could for example be the case for drug efflux pumps which use the proton gradient (and thus indirectly ATP that is used by the cell to maintain the proton gradient) to remove the drug from the cytoplasm. In case of the lac operon, it has been shown by Eames and Kortemme (2012) that the activity of the lactose permease LacY (a proton symporter) is the main driver of the costs of the lac system.
Furthermore, additional transmembrane proteins might allow leaking of protons through the membrane (Stoebel et al., 2008) and space in the membrane might also be limiting, leading to additional costs (Lindén et al., 2012; Dykuizen and Davies, 1980). Resistance genes thus can have wide-ranging effects on other traits, including those that we expect to influence the spread and persistence of resistance such as growth rate and yield.

Biological costs and benefits are central to understanding the persistence of antibiotic resistance genes. When the costs outweigh the benefits of resistance, as is likely in the absence of the drug, resistance should be selected against and eventually disappear. This suggests that a relatively simple cycling strategy, where certain antibiotics would be removed from use for a time, with the intention of reducing the positive selection for resistance, would allow for resistance frequencies to this particular drug to decline. However, studies that look at large scale reductions in antibiotic use are inconclusive, reporting that resistance frequencies only sometimes decline in these cases (Seppälä et al., 1997; Enne et al., 2001; Arason et al., 2002; Bean et al., 2005; Gottesman et al., 2009; Sundqvist et al., 2010; Schechner et al., 2013). Together with direct evidence from lab studies (e.g. Trindade et al., 2012; Hall, 2013; Hall et al., 2011) this shows that these biological costs lead to different outcomes depending on the environment. In order to be able to predict, and possible control, the spread and persistence of antibiotic resistance we need to better understand these biological costs and benefits and how they are modulated by the bacteria themselves and the biotic and abiotic environment.

How do biological costs depend on the environment? If energy and building blocks are present in excess and growth is limited by some other process, using additional energy or building blocks might not influence growth rate or yield. In variable environments costs of resistance will thus vary with the environment. Environments outside the controlled environment of the lab will vary to large extents both temporally and spatially (Wang and Levin, 2009), both
GENERAL INTRODUCTION

on a large scale (e.g. seasonal changes or movement to a different host) and small scale (e.g. diurnal cycles or movement between compartments). Antibiotic treatment can also be seen as a sudden change in the environment, especially when considering the switch to high concentrations at the onset of treatment. The challenge for resistant bacteria is then to keep the costs as low as possible in the absence of drug while still being ready for the, possibly sudden, appearance of the drug.

Gene regulation can help cells cope with these variable environments (Poelwijk et al., 2011) and it has been shown, that protein expression levels have evolved to maximize fitness in a given environment (Liebermeister et al., 2004; Dekel and Alon, 2005). Some antibiotic resistance genes are not expressed constitutively, but are induced by the antibiotic itself (Horinouchi et al., 1983; Hillen et al., 1983; Lindberg and Normark, 1986; Jacobs et al., 1997; Walsh, 2000; Mitrophanov et al., 2008). However, direct regulation seems to be the exception rather than the rule (Price et al., 2013), but is common in horizontally transferred resistance genes (Depardieu et al., 2007). Naively, one would expect that costly resistance genes should be regulated such that the costs only come to bear when the benefits can be realized. It is thus interesting to ask why direct regulation is the exception. Generally speaking, inducible expression is only beneficial if the response time is shorter than the rate of environmental change (Kalisky et al., 2007). Because exposure to antibiotics at high levels is often sudden, this might limit the possibilities for regulation of resistance gene. In Chapter 3 we take a closer look at how populations of inducibly tetracycline-resistant bacteria get around this problem.

This thesis investigates how bacteria manage costly resistance genes (Chapter 2 and Chapter 3) and how biological costs of resistance influence (Chapter 1) and are influenced by evolutionary processes (Chapter 4).
Thesis outline

In **Chapter 1** we make the case for considering costs of resistance on two levels. By distinguishing between effects on traits, such as growth rate and yield, and effects on genotype frequencies, we argue that environmental variation of costs of resistance can be better understood. We show that the same *trait effects* can have different *selective effects* depending on the environment, especially when considering spatial structure.

In **Chapter 2** we take an in depth look at a specific resistance gene, the tetracycline efflux pump *tetA*, and how it is regulated to manage the costs and benefits of resistance. Using a synthetic expression system we quantify costs and benefits at different expression levels of *tetA* and in the presence of different concentrations of tetracycline. We show that the benefits of expressing *tetA* are dependent both on the expression level and on the concentration of tetracycline. Our findings also suggest, that the source of the costs of *tetA* stem from inefficient use of available resources when TetA is present in the cell.

**Chapter 3** addresses an issue that regulation of antibiotic resistance genes can entail. If the resistance gene is completely suppressed in the absence of the drug, there could be situations were cells, albeit genetically resistant, cannot survive the sudden appearance of the drug because upregulation and production of the necessary proteins might be too slow, or even affected by the drug itself, to counteract the effects of the drug. We investigate whether this is the case for the *tetRA* operon also studied in **Chapter 2**. We consider two possible hypotheses how cells carrying *tetRA* can survive the sudden exposure to tetracycline: *i*) after exposure individual cells manage to slowly produce the necessary resistance protein and start to divide again only after a prolonged lag phase. *ii*) a subpopulation manages to continue to divide immediately after exposure. Both hypotheses predict that there will be no population growth for a while but that the growth characteristics on a single cell level should be different. Using time-lapse fluorescence microscopy of a microfluidic device.
we monitor division events and expression of *tetA* in genetically resistant individual bacteria before and after the sudden exposure to tetracycline. Our findings show that there are in fact many genetically resistant cells that fail to divide after sudden exposure to tetracycline while the others continue to divide without a significant lag phase.

Chapter 4 is concerned with effects of evolutionary history on the costs of antibiotic resistance. We experimentally evolved rifampicin resistant and sensitive bacteria in the absence of drugs and then measured competitive costs of new resistance elements in those strains. We find that streptomycin resistance mutations as well as a plasmid carrying a streptomycin resistance gene were less costly in rifampicin resistant strains that were experimentally evolved in the absence of drug. This epistasis between resistance mutations and mutations acquired in the absence of drug might be an important factor that has to be considered when trying to predict the likelihood of the persistence of multidrug-resistant bacteria.
References


Chapter 1

Costs of antibiotic resistance – separating trait effects and selective effects

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Abstract

Antibiotic resistance can impair bacterial growth or competitive ability in the absence of antibiotics, frequently referred to as a ‘costs’ of resistance. Theory and experiments emphasize the importance of such effects for the distribution of resistance in pathogenic populations. However, recent work shows that costs of resistance are highly variable depending on environmental factors such as nutrient supply and population structure, as well as genetic factors including the mechanism of resistance and genetic background. Here we suggest that such variation can be better understood by distinguishing between the effects of resistance mechanisms on individual traits such as growth rate or yield (‘trait effects’), and effects on genotype frequencies over time (‘selective effects’). We first give a brief overview of the biological basis of costs of resistance and how trait effects may translate to selective effects in different environmental conditions. We then review empirical evidence of genetic and environmental variation of both types of effects and how such variation may be understood by combining molecular microbiological information with concepts from evolution and ecology. Ultimately, disentangling different types of costs may permit the identification of interventions that maximize the cost of resistance and therefore accelerate its decline.
1.1 Introduction

Bacterial resistance to antibiotics impairs our capacity to treat infections, posing a growing challenge for global public health (Levy and Marshall, 2004; Bergstrom and Feldgarden, 2008; Smith and Coast, 2013). From the bacterial perspective, resistance is highly advantageous in the presence of antibiotics. However, resistance mechanisms can also impair cellular functions, in turn affecting phenotypic traits such as growth and survival relative to sensitive genotypes in the absence of antibiotics (‘trait effects’ - Box 1). These trait effects can lead to changes in allele frequencies over time (‘selective effect’ - Box 1). For example, if a resistance mutation or plasmid causes bacteria to grow and divide at a slower rate in the absence of antibiotics, this can result in a decrease in the frequency of bacteria with this genotype in a population. Thus, selective effects can be quantified experimentally by monitoring genotype frequencies in populations containing bacteria with and without resistance alleles (Lenski et al., 1991; Trindade et al., 2009; Chevin, 2011; Gullberg et al., 2011). In many studies costs of resistance are defined more loosely, often referring to an effect on a particular phenotypic trait, such as doubling time during exponential growth or stationary phase population density in pure culture (Nagaev et al., 2001; Nilsson et al., 2006; MacLean and Buckling, 2009; Paulander et al., 2009; Petersen et al., 2009; Hall et al., 2011). Here we refer to a cost of resistance strictly as an effect on allele frequencies, that is, a selective effect rather than a trait effect. The motivation for this review is the notion that a clearer distinction between effects on individual traits such as growth rate or yield (trait effects) and how those effects translate to changes of allele frequencies over time (selective effects) may improve our ability to explain variation of costs of resistance.
### Box 1. Glossary

**Resistance mechanism**

A physiological process that increases bacterial growth or survival relative to isogenic bacteria lacking the resistance mechanism at concentrations of antibiotics that reduce growth or survival of the latter. Resistance mechanisms include drug efflux, enzymatic modification and drug-target binding inhibition (Walsh, 2000). The proteins involved in resistance mechanisms are frequently encoded on mobile genetic elements including plasmids and integrons or by specific alleles of chromosomal genes.

**Resistance allele**

A variant of a genetic element that results in expression of a resistance mechanism. For example, several alternative mutations in rpoB can confer resistance to rifampicin in *Escherichia coli* (Garibyan et al., 2003); the specific nucleotide substitution resulting in increased resistance is the resistance allele, and the resistance mechanism is drug-target binding inhibition (Trinh et al., 2006; Sezonov et al., 2007). If resistance is encoded by an entire genetic element that is absent in sensitive cells, such as a plasmid, then we may consider the presence/absence of the plasmid to be alternative ‘alleles’.

**Trait effect**

Change in a phenotypic trait resulting from the presence of a resistance allele. Resistance alleles will frequently affect multiple traits at different levels of organization, and those effects can vary considerably depending on environmental conditions (Section 1.2-1.3) and genetic background, including other resistance alleles or compensatory mutations (Section 1.4).
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Box 1. Glossary (continued)

Selective effect
change in the frequency of a resistance allele in a population over time due to differential survival and reproduction relative to other genotypes, commonly expressed as a selection coefficient \((s)\) \textit{in vitro} or competitive index \((CI)\) \textit{in vivo}. That is, a resistance allele with a negative selective effect will decline in frequency relative to genotypes in the same population lacking the resistance allele. Selective effects can be expressed at any concentration of antibiotics. We note that a change in allele frequencies does not always indicate selection; random processes such as genetic drift can also alter allele frequencies.

Cost of resistance
synonymous with a negative selective effect in this review. In the literature ‘cost of resistance’ has been used to refer to effects on individual traits such as growth rate or yield (typically estimated experimentally by doubling time during exponential growth and population size at stationary phase respectively).

The selective effect of resistance alleles in drug-free conditions is a key determinant of the long-term stability of resistance in pathogenic populations \cite{Andersson and Levin 1999, Levin 2001, Andersson 2003, Cohen et al. 2003, Andersson and Hughes 2010}. For simplicity we focus on costs of resistance in drug-free conditions, but note that a complete understanding of selection on resistance alleles would incorporate their selective effects across a wide range of drug concentrations, because drug concentrations in nature may vary continuously, rather than categorically, over space and time \cite{Baquero and Negri 1997, Hermsen et al. 2012}. Improved understanding of
selective effects potentially enables better management of resistance. For example, if resistance is under negative selection in the absence of drugs, a simple way to reduce resistance would be to reduce antibiotic consumption. However, in cases where use of specific antibiotics has been scaled back, resistance sometimes declines and sometimes does not (Seppälä et al., 1997; Enne et al., 2001; Arason et al., 2002; Bean et al., 2005; Gottesman et al., 2009; Sundqvist et al., 2010; Schechner et al., 2013). This indicates that selective effects of resistance are variable. Consistent with this, experiments show that both trait effects and selective effects—and thus the costs of resistance—vary depending on environmental factors (Table 1.1). For example, the same resistance mutation may be under negative selection in one type of antibiotic-free growth medium and positive selection in another (Trindade et al., 2012). This makes it difficult to predict the selective effect of a given resistance mechanism outside the laboratory. Trait effects and their resultant selective effects can also vary depending on other alleles in the same genetic background, such as those conferring resistance to other antibiotics (Trindade et al., 2009; Andersson and Hughes, 2011), compensatory mutations that epistatically buffer the effects of resistance alleles (Schrag et al., 1997; Levin et al., 2000; Reynolds, 2000; Maisnier-Patin et al., 2002; Maisnier-Patin and Andersson, 2004; Kim and Wei, 2007; Maisnier-Patin et al., 2007; Hall et al., 2010), or regulatory mechanisms that alter the expression of resistance mechanisms in different conditions (Martínez and Rojo, 2011).

Here we ask whether variation in the evolutionary dynamics of antibiotic resistance can be understood by discriminating effects at the level of individual phenotypic traits and at the level of allele frequencies (Fig. 1.1). This approach potentially allows identification of resistance mechanisms or alleles that are consistently under negative selection in different environmental conditions or genetic backgrounds. This is relevant to the management of resistance in pathogenic populations. For example, resistance mechanisms or alleles that are consistently under negative selection in drug-free conditions may
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<td>Nal, Rif, Str</td>
<td>Competition</td>
<td>Nutrients, temperature</td>
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<td>Growth rate</td>
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<td>Growth yield</td>
<td>Carbon source</td>
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<td>Mouse nasopharynx/lung/in vitro</td>
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**Table 1.1** Environmental variation of trait (growth rate or growth yield measured by pure culture growth assays) and selective (competitions) effects. Each study demonstrates variation of the effects of resistance alleles depending on experimental conditions (given under ‘Environmental variation’).
be managed by scaling back antibiotic usage, but this will be less effective for resistance mechanisms that are not consistently under negative selection in drug-free conditions. The information required to link resistance evolution in the laboratory to real-world epidemiological dynamics of resistance determinants is increasingly available, partly because DNA sequencing technology now permits the genetic basis of resistance to be identified in individual outbreaks or chronic infections, allowing not only mechanisms but specific alleles to be monitored (Brockhurst et al. 2011; Snitkin et al. 2012; Palmer and Kishony 2013).

![Diagram](image)

**Fig. 1.1** Effects of resistance alleles at the level of individual traits and allele frequencies. The trait effects depicted here, and consequently their selective effects, can also vary depending on genetic factors, such as the presence of compensatory mutations or other resistance alleles.

### 1.2 Biological basis of costs of resistance

From the perspective of reducing resistance, the most relevant costs are those that ultimately manifest as a decrease in the frequency of resistant bacteria in a population or metapopulation, that is,
negative selective effects. What biological processes are responsible for the changes in phenotypic traits that ultimately lead to such a change in the composition of a population? Two ways how resistance mechanisms can incur such selective effects are quite obvious: they can slow down cellular functions and thus decrease the rate at which bacteria grow and divide, or they can divert building blocks or energy from the growth of biomass, and thus lower yield, that is, the number of bacteria that emerge out of a fixed amount of resources. These two scenarios are not mutually exclusive; some resistance mechanisms may have correlated effects on both rate and yield \cite{Fitzsimmons2010}. These reductions in growth rate or yield or both can then result in a decreased frequency of resistant bacteria in a population. The focus on rate and yield is a simplification, since other biological traits will also influence the dynamics of genotype frequencies. For example, survival during exponential growth or after cessation of growth and entry into stationary phase can differ between genotypes, and will translate into selective effects. Reduced survival during exponential growth, for example, will translate into a reduction in the net growth rate of a clonal population. A broader definition of growth rate and yield can incorporate contributions of other traits. In Section 1.3 we come back to the issue of trait effects other than rate and yield that lead to selective effects, and discuss how resistance mechanisms can impact gene expression and regulatory responses to stressful conditions.

These considerations make it evident that costs of resistance are inherently context-dependent. This is for two reasons. First, whether a change in a cellular function impacts growth rate or yield depends on external conditions. In some conditions, a given function might limit the rate at which bacteria grow, or the yield they achieve. In other conditions, the same function might not limit rate or yield, and a reduction in this function due to the presence of an antibiotic resistance allele will thus not affect these two traits. Second, whether a reduction in growth rate or yield impact the frequency of the resistant type over time depends on the population structure and whether
**Fig. 1.2** Selective effects of alleles, such as antibiotic-resistance alleles in the absence of drugs, that reduce growth rate (“slow” — A, B) or yield (“inefficient” — C, D) in unstructured (A, C) and structured (B, D) populations. The wild type converts one unit of resource r to produce one additional cell and growth continues, with no cell death, until all resources are depleted. ‘Slow’ bacteria produce new cells at a lower rate than the wild type but with the same efficiency, causing them to decline in frequency in spatially unstructured conditions where resources are shared. “Inefficient” bacteria produce new cells at the same rate but use $2 \times r$ per new cell, declining in frequency across a spatially structured metapopulation where clonal demes consume resources “privately” ([Pfeiffer et al.], 2001; [Bachmann et al.], 2013).
growth resources are shared or private (Pfeiffer et al., 2001; MacLean and Gudelj, 2006; Frank, 2010, 2014). In well-mixed environments with shared resources, genotype frequencies over time are mostly dependent on growth rate. In other conditions, for example when clonal populations inhabit patches with limited dispersal, genotype frequencies, expressed as a fraction of the total metapopulation, can depend mostly on yield (Fig. 1.2). This has been demonstrated experimentally in populations of *Lactococcus lactis* (Bachmann et al., 2013). This study, like Fig. 1.2, assumed no migration among patches. In reality, spatially structured populations will often be subject to some degree of migration and genetic mixing. Theory suggests that as mixing increases, greater resource competition shifts the balance toward high-rate, rather than high-yield, phenotypes (Pfeiffer et al., 2001; Frank, 2010). Based on these considerations, we would predict changes in the costs of resistance across external conditions to be the norm rather than the exception.

In order to explain observed variation in costs of resistance, we suggest breaking the problem of identifying costs in two parts. The first part describes how a given resistance mechanism affects the rate at which bacteria grow or the yield they achieve. Because changes in rate and yield may be determined by trait effects at other levels of organization (Section 1.3), such inference can be based on molecular microbiological information (Ruusala et al., 1984; Brandis et al., 2012), metabolic control analysis (Dykhuizen and Dean, 1990; Fell and Cornish-Bowden, 1997) or related approaches that describe how the growth characteristics of an organism depend on external factors such as availability of limiting nutrients (Mortlock, 1984; Lendenmann et al., 1996). The second part is then to analyze how trait effects influence the frequency and total abundance of the resistant type in a population or metapopulation. Note that in certain conditions changes in rate or yield may alter population-level growth without affecting allele frequencies, such as a reduced yield in spatially non-structured conditions (Fig. 1.2C). This analysis can be based on a well-developed body of theory on how the action
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To make these considerations more concrete let us consider the specific example of a bacterium that carries a gene encoding a β-lactamase conferring resistance to penicillins. What are the ‘costs’ of this resistance mechanism? Let us first focus on trait effects. An important distinction is whether the β-lactamase is constitutively expressed or whether expression is induced by the presence of antibiotics (Minami et al. 1980). In the latter case, bacteria that grow in the absence of antibiotics will produce no, or only very little, of the β-lactamase protein. The presence of the gene itself might still have cellular consequences that could manifest as marginal costs. Under conditions where the doubling time of the bacterium is limited by the time needed to replicate the chromosome, the presence of an extra gene on the chromosome will increase the time, although only slightly so (if the replication time is proportional to the length of the chromosome, then the increase will be about 0.1% per additional gene for a bacterium with a chromosome of 1000 genes, and even smaller for bacteria with more genes). Likewise, the extra building blocks required for the increased size of the chromosome are expected to be marginal. The resources required for DNA are a small fraction of a cell’s total resources, and an increase in DNA content resulting from the addition of one gene thus has small consequences (Brinas et al. 2003).

The main trait effects are therefore expected to result from expression of the gene and production of the β-lactamase protein, resulting from constitutive expression or environmental induction of expression (Dekel and Alon 2005, Stoebel et al. 2008). What are these effects? The production of a protein requires building blocks assimilated from carbon and nitrogen, and energy. If any of these three resources are limiting the growth rate or yield of bacteria, in that an increase or decrease in the resource causes a concordant increase or decrease in rate or yield, then expressing the β-lactamase will reduce the trait in question, and the magnitude of this reduction will depend
on the amount of resistance protein produced relative to the total biomass of a cell. Alternatively, it is conceivable (and realistic for many natural situations) that growth rate and yield are limited by other elements, for example iron or sulfur (Joyner and Lindow 2000; Mann and Chisholm 2000; Gourion et al. 2006). In these cases, one would expect that investing carbon, nitrogen and energy into producing β-lactamases would have virtually no consequences for a bacterium’s growth traits, with the caveat that investing these resources into antibiotic resistance could lead to reduced investment into the machinery for the acquisition of iron and sulfur, or expression of the protein itself may pleiotropically affect other traits, and thus indirectly reduce the growth rate or yield.

Let us now consider conditions where the growth yield of the bacterium is limited by nitrogen, and where the production of large amounts of β-lactamase diverts nitrogen away from the production of cellular biomass, reducing the number of bacteria that can be formed from a given amount of resources, that is, the yield. How this trait effect translates into a selective effect, that is, how it influences the frequency of the resistant type, depends on the population structure. Specifically, if resistant and sensitive bacteria compete in a well-mixed environment, then the reduced yield of the resistant bacterium is not expected to result in a competitive disadvantage (Pfeiffer et al. 2001; Frank 2010). The reduction in available nitrogen resulting from the production of β-lactamase affects both types equally, and does not lead to a competitive imbalance. By contrast, if resources occur in patches that are colonized by one or a few individuals that expand into clonal populations before they disperse again, then the yield that a clone achieves can be decisive for its competitive success (Fig. 1.2). We will revisit context-dependent costs below, and discuss how these considerations help explain previously observed variation in the measured costs of resistance.
1.3 Environmental variation of trait and selective effects

Consistent with the above rationale that trait and selective effects are context-dependent, several independent experiments with different types of antibiotic-resistant bacteria show that the phenotypic effects of resistance alleles and their influence on allele frequencies over time vary strongly depending on environment (Table 1.1). In these studies trait effects such as changes in growth rate or yield and selective effects as inferred by competition assays are both referred to as ‘costs of resistance’. We suggest that, particularly given environmental variation of these effects, distinguishing trait and selective effects is beneficial.

For example, many in vitro investigations of ‘costs’ in different conditions are based on growth rate measurements (Nagaev et al., 2001; Nilsson et al., 2006; MacLean and Buckling, 2009; Paulander et al., 2009; Petersen et al., 2009; Hall et al., 2011). They are usually performed in well-mixed conditions in liquid culture, where a reduction in growth rate is likely to translate to a negative selective effect. That is, in these conditions the trait effect (change in growth rate) gives a reliable indication of the likely selective effect of resistance, so the failure to distinguish the two is not critical. Consistent with this, some studies have demonstrated a positive association between effects of resistance mechanisms in growth rate assays and pairwise competitions (Petersen et al., 2009; Perron et al., 2010; Guo et al., 2012). However, if the same type of experiments were done in spatially structured environments, selective effects may be more sensitive to changes in yield than growth rate (Kreft, 2004; Bachmann et al., 2013). In such conditions changes in maximum growth rate are no longer a reliable indicator of selective effects, and discriminating the two types of effect is more important for understanding the incidence of resistance over time. Crucially, growth in spatially structured environments with distinct subpopulations is probably more realistic than growth in well-mixed environments for many pathogenic species.
Therefore if we aim to investigate costs of resistance across realistic settings it is potentially misleading to group both types of effect under the same term, given that the same trait effect may translate to different selective effects in different environments.

The relevance of this problem is demonstrated by comparing selective effects estimated for the same genotypes \textit{in vitro} and \textit{in vivo}. Several experimental studies [Björkman \textit{et al.}, 1998; Nilsson \textit{et al.}, 2004; Marcusson \textit{et al.}, 2009], as well as a meta-analysis (Vogwill and MacLean, 2015), indicate a positive overall association: resistance alleles that are under negative selection \textit{in vitro} tend to also be under negative selection \textit{in vivo}. However, in other studies the association is weaker, and the rank order of different resistance alleles in terms of their selective effects can differ between assay conditions (Table 1.1). A key factor in determining the association between \textit{in vitro} and \textit{in vivo} results appears to be the mechanism of antibiotic resistance. For example, data presented by Björkman \textit{et al.} (2000) show a strong positive correlation between \textit{in vitro} and \textit{in vivo} selective effects for various streptomycin-resistance alleles ($r^2 = 0.56, P = 0.02$), but no significant correlation for fusidic acid-resistance alleles ($r^2 = 0.13, P = 0.13$). The lack of association here could be due to variation of the trait effects of individual alleles across assay conditions, or because the same trait effects result in different selective effects \textit{in vitro} and \textit{in vivo}. For instance, a mouse is more spatially structured compared to a shaken test tube or microplate, and therefore selective effects \textit{in vivo} may be more sensitive to changes in yield than rate. We note that \textit{in vivo} and \textit{in vitro} conditions will also differ in other ways such as nutrient availability and interaction with the host immune system, which could also modify trait and selective effects. In such scenarios our understanding of variation of selective effects across conditions may be improved by examining the effects of specific resistance alleles on individual traits like growth rate and yield, and testing their correlation with selective effects observed in competition experiments.

We have so far focused on trait effects at the level of growth param-
eters such as rate and yield, because these are the most frequently measured and are the ultimate basis of selective effects. However, the bacterial phenotype is composed of a vast number of traits. Resistance alleles may have multiple trait effects at different levels of organization, and those trait effects may vary environmentally. For example, streptomycin-resistance mutations on \textit{rpsL} directly impair translation, which reduces growth rate and has a negative selective effect in rich growth media \cite{Kurland1992}. However, the same mutations repress induction of a stress-associated $\sigma$-factor in minimal media containing poor carbon sources, resulting in a dampened stress response and rapid growth compared to the wild type \cite{Paulander2009}. Altered stress responses are also implicated in the increased survival of streptomycin-resistant mutants inside macrophages \cite{Miskinyte2013}. Thus, a predictive understanding of the effects of resistance alleles on bacterial growth parameters may be gained by quantifying their effects on specific processes, in this case translation and stress response induction, in different conditions.

Resistance alleles that pleiotropically affect multiple traits are probably very common. Mutations that confer antibiotic resistance by modifying the cellular target of an antibiotic typically occur on genes encoding proteins involved in essential functions for cellular growth and survival \cite{Walsh2000}. As in the example of \textit{rpsL} mutations above, such mutations can have wide-ranging consequences for the expression of other genes or the activity of pathways that are not directly related to the function of the mutated enzyme. If the influence of these pleiotropic effects on growth rate or yield varies environmentally, this may result in variation of the selective effects of resistance alleles across environments, even if the relationship between rate or yield and selective effects is constant. This is illustrated by the observation that rifampicin-resistance mutations on RNA polymerase can influence expression across the entire genome \cite{Applebee2008, Conrad2010, Derewacz2013}, and pleiotropically influence other traits that are not directly linked to transcription,
such as metabolism of carbon sources (Jin and Gross 1989; Perkins and Nicholson 2008; Paulander et al. 2009). As a result, rifampicin-resistance mutations can be positively selected in some antibiotic-free conditions, such as adaptation to novel carbon sources (Applebee et al. 2008; Conrad et al. 2010; Tenaillon et al. 2012), aging colonies (Wrande et al. 2008) or high temperature (Rodríguez-Verdugo et al. 2013).

Alleles that confer resistance via efflux pumps also tend to have pleiotropic effects, because they often have broad activities against antibiotics and other compounds (Nikaido 1998; Piddock 2006; Nikaido and Pagès 2012). This is relevant for the distribution of resistance in pathogenic populations because a single allele encoding resistance against multiple antibiotics permits coselection, where positive selection for resistance to one drug causes resistance to other drugs to spread. Indeed, coselection has been implicated in the persistence of some resistance mechanisms, such as trimethoprim resistance in Escherichia coli in Sweden (Sundqvist et al. 2010) and sulfonamide resistance in E. coli in the United Kingdom (Enne et al. 2001; Bean et al. 2005), despite restrictions on use of these antibiotics. Coselection of resistance alleles may also result from selection for resistance to heavy metals present as contaminants in the environment (Baker-Austin et al. 2006). In scenarios where coselection is possible, such as populations that are exposed to different drugs over time, the selective effects of resistance alleles across conditions are best understood by quantifying trait effects in terms of resistance to multiple compounds. Recently, this approach has been used to predict the effects of different interventions by screening for cross-resistance and collateral sensitivity among clinically relevant combinations of drugs (D’Costa et al. 2006; Imamovic and Sommer 2013; Lázár et al. 2013).

Coselection can also occur when multiple resistance alleles occur on the same genetic background. For example, plasmids and resistance gene cassettes often encode resistance against multiple antibiotics on separate genes (Alekshun and Levy 2007; Chambers and Deleo...
Multi-drug resistance can also be acquired via sequential acquisition of different resistance mutations on chromosomal genes (Livermore 2002; Da Silva and Palomino 2011). Interestingly, in cases where multiple resistance alleles are present on the same genome, plasmid or lineage of cells, their net trait effects and resultant selective effects in combination may deviate from what we would predict based on their independent effects. Therefore understanding the potential for coselection requires that we consider epistatic variation of trait and selective effects. More generally, variation of trait or selective effects depending on genetic background (epistasis) constrains our ability to translate in vitro results to real-world scenarios, because evolving pathogenic populations will differ genetically from laboratory strains. We next discuss whether this type of variation can also be understood by distinguishing trait effects and selective effects of resistance alleles.

1.4 Epistatic variation of trait and selective effects

Recent work shows that the trait effects of resistance alleles, expressed as changes in growth rate or yield in the absence of antibiotics (Ward et al. 2009; Hall and MacLean 2011) or at inhibitory antibiotic concentrations (Weinreich et al. 2006; Salverda et al. 2011) vary strongly depending on the presence of other resistance alleles on the same genetic background. Furthermore, studies that have measured selective effects in vitro through competition assays in the absence of antibiotics have revealed pervasive epistasis between resistance alleles (Rozen et al. 2007; Trindade et al. 2009; Silva et al. 2011). That is, the same resistance allele may have different selective effects across genetic backgrounds that vary at other loci involved in antibiotic resistance. These interactions are relevant for understanding resistance in pathogenic populations. For example, recent work shows that combinations of mutations associated with a small or no negative selective effect in the absence of antibiotics in vitro appear to be
overrepresented among clinical isolates of *Mycobacterium tuberculosis* (Borrell *et al.*, 2013), indicating that laboratory measurements can be predictive of evolutionary dynamics in natural hosts. In some scenarios the net selective effect of multiple resistance alleles may be understood by considering how the trait effects of one allele can be influenced by the presence of another resistance allele.

For example, the negative selective effects in drug-free conditions of rifampicin-resistance mutations on *rpoB* tend to be buffered by the presence of streptomycin-resistance mutations on *rpsL* (Trindade *et al.*, 2009). Independently, these resistance mechanisms impair transcription and translation respectively (Kurland 1992; Reynolds 2000), and this is associated with reduced growth rate. However, molecular studies show that activity of these enzymes is very closely related (Dutta *et al.*, 2011), and impairment of one can indirectly inhibit the other (Proshkin *et al.*, 2010), effectively placing a speed limit on the transcription-translation pathway. Thus, the influence of an *rpoB* mutation on transcription and bacterial growth rate may be relatively small in conditions where ribosomal activity is inhibited. Consistent with this, the negative effects of *rpoB* mutations on bacterial growth rate can be reduced by the addition of ribosome inhibitors (Hall *et al.*, 2011). In such conditions reduced growth rates typically translate to negative selective effects (Vogwill and MacLean 2015), so the prevalence of antagonistic epistasis between *rpoB* and *rpsL* mutations in their selective effects (Trindade *et al.*, 2009) may be explained by antagonism between their effects on growth rate (Ward *et al.*, 2009). It is not yet known whether their effects on transcription and translation also interact epistatically. Nevertheless, combining molecular microbiological information with measurement of trait and selective effects could also be applied to other combinations of resistance mechanisms, because the molecular basis of resistance is known for many combinations of bacteria and drugs.

Epistatic interactions have also been observed among resistance mutations on the same gene. For example, in a β-lactamase, epistatic interactions in terms of mutational effects on resistance against
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Cefotaxime have been explained by examining interactive changes in enzyme activity and thermodynamic stability (Salverda et al., 2011; Schenk et al., 2013) (see also Bershtein et al., 2006). Given that almost all mutations affect stability (DePristo et al., 2005; Tokuriki et al., 2007), and that stability is a key determinant of the effective concentration of functional enzymes in a cell (Pakula and Sauer, 1989), the same approach could be applied to understand the physiological basis of trait effects in the absence of antibiotics for resistance mechanisms that involve multiple mutations on the same chromosomal gene, such as fluoroquinolone resistance in E. coli (Komp Lindgren et al., 2003; Lindgren et al., 2005; Marcusson et al., 2009). For detailed reviews of the mechanistic drivers of epistasis see Lehner (2011) or de Visser et al. (2011).

Epistasis at the level of trait and selective effects may also influence the distribution of antibiotic resistance in scenarios where a resistance allele modulates the effects of mutations at other sites that are not directly involved in resistance mechanisms, such as compensatory mutations that have a positive selective effect only for genotypes with resistance alleles. Compensatory mutations can modify the effect of various resistance mechanisms [reviewed by Maisnier-Patin and Andersson (2004) both in vitro (Schrag et al., 1997; Maisnier-Patin et al., 2002; Brandis et al., 2012) and in vivo (Björkman et al., 2000)]. There is mounting evidence that compensatory mutations are not only quickly selected in laboratory settings, but are common in resistant clinical isolates (Shcherbakov et al., 2010; Comas et al., 2012; de Vos et al., 2013). Therefore even in cases where the effects of a resistance allele on growth traits are understood, their influence on the effects of mutations at other loci should also be considered.

The trait effects of alleles at other loci can also help to identify the trait effects of the original resistance allele that are under negative selection in the absence of antibiotics, that is, the physiological basis of costs of resistance. For example, expression of some resistance genes is induced by the antibiotic itself (Minami et al., 1980; Depardieu et al., 2007; Foucault et al., 2010). The fact that such reg-
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Regulatory mechanisms have evolved indicates that spurious expression (transcription and translation) of resistance genes in antibiotic-free conditions has a negative selective effect. Consistent with this, induction of expression of tetA in the absence of tetracycline incurs high costs in competition experiments in E. coli K12 (Nguyen et al. 1989), while without induction the resistance allele has no measurable selective effect. Similarly, vancomycin resistance has a negative selective effect when expression is induced in the absence of antibiotics both in vivo and in vitro (Foucault et al. 2010). Therefore analysis of the trait effects of alleles at other loci, including regulatory mechanisms and compensatory mutations, may provide insight into the biological basis of the cost of the original resistance allele.

1.5 Conclusions and future directions

The take-home message of this review is that variation of ‘costs of resistance’ can be better understood by distinguishing effects on individual traits and on genotype frequencies over time. It is unnecessarily misleading to group changes in growth rate or yield in pure culture and selective effects inferred from in vitro or in vivo competitions all under the same term. The motivation and justification for investigating ‘costs of resistance’ is typically to gain insight into the likelihood that resistance will persist or decline in the absence of antibiotics. In a given environment this is defined by selective effects as inferred from changes in genotype frequencies, rather than trait effects such as changes in growth rate or yield. For many types of resistance and environmental conditions measured trait effects such as growth rate are strong predictors of selective effects, but this is not always the case. In some cases resistance alleles may have important trait effects that do not result in a selective effect, such as reduced yield in spatially non-structured conditions (Fig. 1.2C). Therefore we do not suggest that trait effects are unimportant or that selective effects are the only relevant parameter for managing resistant infections, only that disentangling the effects of resistance
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alleles at different levels of organization will be beneficial and that using more specific terminology would be a good start.

Such information can potentially be applied to the management of resistance. For example, when the physiological basis of selective effects is understood, novel strategies may be devised to maximize costs by environmental manipulation, ultimately accelerating the decline of resistance. In the case of resistance genes associated with regulatory mechanisms, one way to achieve this would be via compounds that induce costly expression of resistance genes in the absence of antibiotics, such as analogues of relevant drugs (Nguyen et al., 1989). A similar approach is plausible for resistance alleles on chromosomal genes, such as streptomycin-resistance mutations on \( rpsL \) or rifampicin-resistance mutations on \( rpoB \). In these examples the knowledge that selective effects are often related to defective translation or transcription (Kurland, 1992; Reynolds, 2000) suggests that conditions where the rate of gene expression limits growth rate, and selective effects are correlated with growth rate effects, are less likely to sustain resistance in the absence of antibiotics. Such conditions might be artificially created using signalling molecules that induce expression of the many quorum-sensing-regulated genes, typically acyl-homoserine lactones for Gram-negative and processed oligo-peptides for Gram-positive bacteria (Whiteley et al., 1999; Miller and Bassler, 2001; Hall et al., 2011), thereby increasing the total number of genes expressed and potentially increasing the likelihood that impaired transcription or translation due to the presence of resistance alleles reduces bacterial growth rate. As a first step, these and other interventions aimed at increasing costs of particular resistance alleles could be investigated \textit{in vitro}.

More generally, understanding whether costs are consistently low or high for a given type of resistance across alternative alleles and environments will help to predict whether restricted usage of drugs will work. We suggest a pluralist approach to this problem, with at least three valuable types of information. First, molecular microbiological information allows relevant trait effects to be identified, as
in the example of \textit{rpsL} mutations that affect both translation and stress response induction (Paulander \textit{et al.} \citeyear{Paulander2009}). Second, comparing the selective effect of the same resistance allele across different conditions, both \textit{in vitro} and \textit{in vivo}, indicates whether trait and selective effects identified in one environment translate to other conditions. Continuing with the example of \textit{rpsL} mutations, the role of altered stress response induction as observed \textit{in vitro} was translated to a clinically relevant environment (macrophages) by Miskinyte and Gordo \citeyear{Miskinyte2013}. In general, the influence of population structure on selective effects of resistance alleles is particularly important given that many pathogenic species grow in spatially structured biofilms \cite{Costerton1995, Kreft2004}. Third, in an epidemiological context, we are concerned not only with selective effects within a given host, but in a population of hosts that are connected, from the pathogen’s perspective, by transmission. Therefore comparing selective effects \textit{in vitro} and \textit{in vivo} to the distribution of the same resistance alleles in collections of clinical isolates provides a key test of how informative experimental evolutionary dynamics are in real pathogenic populations. This approach was recently applied successfully in \textit{Mycobacterium smegmatis} and \textit{tuberculosis} by Borrell \textit{et al.} \citeyear{Borrell2013}.

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Chapter 2

Biological costs and benefits of \textit{tetA}

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Abstract

Biological costs of resistance are important factors for the persistence and spread of antibiotic resistance. Here we investigate how gene expression of dedicated resistance genes can modulate the biological costs and the benefits of antibiotic resistance.

We study the tetracycline resistance gene tetA using a synthetic expression system that allows us to measure biological costs in terms of reduction in growth rate and yield at different expression levels of tetA. We do this both for a construct where expression levels are varied independent of the tetracycline concentration and for a construct that uses the tet promoter which is directly induced by tetracycline itself.

Our results confirm that expression of tetA is costly in the absence of drug. However, these effects are only visible at relatively high expression levels and are more pronounced when considering yield, especially in conditions where yield is carbon limited. This could indicate that the source of these costs stem from inefficient use of available resources. We also show that the benefits of expressing tetA are dependent both on the expression level and on the concentration of tetracycline.

A quantitative understanding of the biological costs of resistance can help us understand better in what conditions resistance is likely to be stable or selected against. Differentiating between effects on growth rate and yield is important when considering possible selective effects of the biological costs.
2.1 Introduction

Biological costs of resistance, as measured by a reduction in a life-history trait such as growth rate or yield, are important factors for the persistence and spread of antibiotic resistance (Levin et al., 1997; Andersson and Hughes, 2011; Abel zur Wiesch et al., 2011; see also Chapter 1). In the absence of drugs, resistance typically incurs a cost (Andersson and Levin, 1999; Björkman and Andersson, 2000; Gagneux et al., 2006; Andersson and Hughes, 2010) while benefits are usually only realized in the presence of the drug. The expectation is therefore that resistance frequencies decrease in the absence of the drug. However, evidence from both laboratory studies (Griffiths et al., 1990; Dahlberg and Chao, 2003; De Gelder et al., 2004) and large scale intervention studies (Enne et al., 2001; Gottesman et al., 2009; Sundqvist et al., 2010), where the use of certain drugs was restricted, show no or only a slight reduction in the frequency of antibiotic resistance.

Most previous work has focused on mutational resistance and the influence of compensatory evolution (Björkman et al., 2000; Shcherbakov et al., 2010), epistasis of resistance mutations (Trindade et al., 2009) and selection of resistance mutations by unrelated stressors (Rodríguez-Verdugo et al., 2013). Here we focus on dedicated resistance genes, such as, for example, efflux pumps. How can dedicated resistance elements persist in the absence of drugs?

One explanation is that there is still selection for these resistance elements, even in the apparent absence of the drug itself. Even very low concentrations of antibiotics can maintain or even select for resistance (Gullberg et al., 2011). Furthermore, coselection can explain persistence of resistance elements that are linked to other elements under selection, such as other antibiotic resistances (Enne et al., 2001) or heavy metals (Seiler and Berendonk, 2012). Another explanation is that fitness cost of antibiotic resistance genes are often reduced by subsequent mutations and are dependent on genetic context. Examples are compensation by additional muta-
tions (Bouma and Lenski, 1988) or epistatic interaction with other resistance elements (Chapter 4).

While a lot of work has been done investigating how bacteria handle or adapt to costs of resistance in the absence of direct selection, less attention has been paid to costs of resistance genes in the presence of drug or when drug concentrations are fluctuating. Costs of resistance vary with drug concentration (Gullberg et al. 2011) and the fate of resistance genes in variable environments will thus also depend on the nature of the relationship between costs of resistance and the concentration of the drug. This relationship can be shaped by the regulation of the genes responsible for resistance.

Resistance genes are often horizontally acquired along with their own regulatory context. Some of these genes are not expressed constitutively, but are induced by the antibiotic itself (Horinouchi et al. 1983; Hillen et al. 1983; Lindberg and Normark 1986; Jacobs et al. 1997; Walsh 2000; Mitrophanov et al. 2008). This specific gene regulation can negate the costs of resistance genes in the absence of drugs (Nguyen et al. 1989; Foucault et al. 2010) while still enabling resistance in the presence of the drug.

Gene regulation is expected to evolve in response to the specific environmental conditions and dynamics the bacteria experience by solving a cost-benefit optimization problem (Dekel and Alon 2005; Poelwijk et al. 2011). The regulation of the resistance gene is thus expected to be tuned such that costs are minimized while benefits are maximized under the present conditions. To understand this problem in a quantitative manner it is necessary to quantify both the costs and the benefits of expression and how they change in a variable environment.

Expression of additional genes generally reduces growth rates, because of the allocation of ribosome away from other genes required for growth (Scott et al. 2010). The presence and expression of a gene in a cell will also entail some metabolic burden (Glick 1995), e.g. by diverting energy or building blocks from growth related processes.
In the absence of expression this metabolic load will presumably be marginal as replication time of an additional gene and the required building blocks are expected to be only a small fraction of the cell's total metabolic requirements (see Chapter 1). The mere presence of the protein will also entail some costs (e.g. for membrane proteins, through disorganization of the inner membrane with associated loss of membrane potential [Eckert and Beck, 1989]). Also, the specific or unspecific activity of the protein could entail some metabolic burden (Eames and Kortemme, 2012). Whether this metabolic burden translates to reductions in growth rate or yield depends on external conditions. This is because the impacted function might not limit these traits in all environments (Chapter 1).

We use the tetracycline resistance operon tetRA to investigate how costs and benefits of a costly resistance gene are mediated across different tetracycline concentrations. We measure costs and benefits as reduction in growth rate or yield. The metabolic burden of tetA could be different in the presence of tetracycline, where more energy might be expended for the activity of the TetA efflux pump, leading to different effects on growth rate and yield. Because these effects will also depend on the environment, we did our experiments in conditions where yield is either limited by the available carbon source or by other nutrients [Bren et al., 2013]. Using both a synthetic construct where expression of tetA can be controlled independent of tetracycline concentration and the full tetRA regulon we aim to answer the following questions: i) How do the benefits relate to the level of expression? ii) Is the optimal level of expression dependent on the tetracycline concentration? and iii) Does the tetRA operon regulate tetA such that it is expressed at the level that maximizes benefits for different tetracycline concentrations?
2.2 Methods

Media and growth conditions

Strains were grown at 37°C in M9 minimal media (Sigma) with 0.2% (w/v) glucose as the sole carbon source or on LB agar (1.5% agar, Sigma). An additional experiment was conducted with 0.02% (w/v) glucose to test for effects on growth and yield under conditions where yield is limited by the carbon source \(^{[1]}\) Bren et al., 2013. L-arabinose (Sigma) was supplemented as indicated. Strains with plasmids were grown with kanamycin at a concentration of 30 \(\mu g/ml\) in both solid and liquid media to maintain the plasmid. Tetracycline (Sigma) stock solutions were prepared in 95% ethanol and diluted into growth medium at the concentrations indicated.

Plasmids and strains

Tetracycline is a bacteriostatic antibiotic that inhibits protein synthesis by reversibly binding to the 30S subunit of the bacterial ribosome, thereby preventing the association of tRNA with the ribosome \(^{[2]}\) Thaker et al., 2010. The inducible resistance operon \(\text{tetRA}\) consists of two genes, one coding for the repressor TetR and the other coding for a tetracycline efflux pump, TetA. The genes are organized in opposing directions and share an overlapping control region. In the absence of tetracycline, the repressor protein TetR binds to two operators between the two genes, thereby efficiently repressing transcription of both itself and \(\text{tetA}\) \(^{[3]}\) Hillen et al., 1983; \(^{[4]}\) Chopra and Roberts, 2001. Expression of \(\text{tetA}\) and \(\text{tetR}\) is induced when tetracycline enters the cell and binds to the repressor, thereby releasing it from the two operators \(^{[5]}\) Chopra and Roberts, 2001. In order to be able to measure the costs and benefits of expression we designed a plasmid borne expression system, referred to as pBtetA-YFP, based on the arabinose inducible promoter pBAD were expression of \(\text{tetA}\) can be controlled by the addition of L-arabinose to the media (Fig. 2.1 and Table 2.1).
The expression system was designed in silico using Geneious version 6.1.8 (Kearse et al., 2012) and is based on a tetA-YFP fusion protein obtained from Magdalena Steinrück at IST Austria. The tetRA sequence was obtained from the NCBI Nucleotide database (GenBank Accession Number: AF162223.1, Chalmers et al., 2000). The sequence of the YFP fluorophore was also taken from the NCBI Nucleotide database (GenBank Accession Number: AJW76798.1). In order to avoid an internal BsaI restriction site in the YFP gene two synonymous mutations were inserted (640A>C and 642A>T). To generate the tetA-YFP fusion the two genes were joined using a 18bp Glycine-Serine linker replacing both the stop codon of tetA and the start codon of the YFP gene. Sequence for the P_{BAD} - araC inducible promoter and the downstream ribosome binding site was obtained from the Registry of Standard Biological
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Parts (Part: BBa_K607036\textsuperscript{4}). All constructs were flanked by modified thr terminators to prevent read through (upstream: BBa_0010\textsuperscript{5} and downstream: BBa_1010\textsuperscript{6}) and a custom cloning site. The control insert consist of the two cloning sites separated by 6 bases plus an additional 71 bases. The bases were chosen randomly and adjusted to maintain the unique restriction sites in the cloning sites. The constructs and the control insert were synthesized by DNA2.0 (Newark, California, USA) and cloned into the high-copy plasmid pJ247 (pBtetA-YFP and pTetRA-YFP) or pJ201 (pControl). Both plasmids have the same pUC origin of replication. All constructs were verified by sequencing by DNA2.0 (Newark, California, USA.). Full sequences of the plasmids are provided in \textbf{APPENDIX A|SECTION A.3}.

The plasmids were all transfered to \textit{Escherichia coli} BW27784 \cite{Khlebnikov2001} using TSS transformation \cite{Chung1989}. \textit{E. coli} BW27784 is unable to utilize arabinose and expresses \textit{araE} constitutively from promoter \textit{P}_{CP18}. This allows for homogeneous expression from the arabinose-inducible promoter \textit{araBAD} \cite{Khlebnikov2001} within the population. BW27784 was obtained from the \textit{E. coli} Genetic Stock Center (CGSC#7881). Hereafter, \textit{E. coli} BW27784 carrying one of the plasmids will be referred to by just the name of the plasmid. All plasmids used are summarized in \textbf{TABLE 2.1}.

**Growth experiments**

Growth experiments with strains carrying one of the three plasmids described above were done in black 384-well polystyrene clear-bottom plates (Greiner 781096). On each plate all combinations of 5 concentrations of tetracycline (16, 8, 4, 2 and 0 µg/ml) and 5 concentrations of L-arabinose (10\textsuperscript{-3}, 10\textsuperscript{-4}, 10\textsuperscript{-5}, 10\textsuperscript{-6} and 0% w/v) were tested for each plasmid for a total of 25 different experimental conditions per

\begin{itemize}
\item \url{parts.igem.org/Part:BBa_K607036} accessed 2015/08/20
\item \url{parts.igem.org/Part:BBa_0010} accessed 2015/08/20
\item \url{parts.igem.org/Part:BBa_1010} accessed 2015/08/20
\end{itemize}
2.2. METHODS

Table 2.1 Plasmids used in this study. All plasmids are used in strain BW27784 (BW25113 Δ(araFGH) Φ(ΔaraEp P_{CP18-araE}) (Khlebnikov et al., 2001)).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>pControl</td>
<td>tetracycline sensitive control</td>
</tr>
<tr>
<td>pBTetA-YFP</td>
<td>L-arabinose inducible expression of tetA-YFP</td>
</tr>
<tr>
<td>pTetRA-YFP</td>
<td>tetA-YFP under control of the original tet promoter</td>
</tr>
</tbody>
</table>

Plasmid. Five biological replicates of each condition for each plasmid were arranged randomly on the plate. The remaining 9 wells were filled with media without any tetracycline and L-arabinose and were used as a sterile control. The whole assay was simultaneously replicated four times on individually randomized plates. The plates were incubated at 37°C at 95% relative humidity to prevent evaporation. Every 30 minutes plates were moved from the incubator to the spectrophotometer, shaken for one minute and optical density at 595nm (OD) was measured. After every OD measurement YFP fluorescence was measured using an excitation wavelength of 485±20nm and an emission wavelength of 535±25nm.

Plate setup and reading of the plates at regular intervals were done using a Tecan Freedom Evo 200 liquid handling platform (Tecan, Männedorf, Switzerland) with an integrated humidity controlled incubator (Liconic, Mauren, Principality of Liechtenstein) and Tecan Infinite F200 Spectrophotometer (Tecan, Männedorf, Switzerland).

Data analysis

The first four OD reads of the plates were discarded due to anomalous readings caused by condensation on the surface of the warm plates when exiting the humidity controlled environment of the incubator.
Background corrected OD\(_c\) was calculated as

\[
OD_c(t) = OD_{raw}(t) - \min(OD_{raw}). \tag{2.1}
\]

for each well. OD\(_c\) values below an arbitrarily chosen cutoff of 0.01 were discarded.

Maximum specific growth rates (\(\mu_{max}\)) were obtained by fitting linear models to a sliding window of 6 log\(_2\)-transformed reads (approximately 3 h). Growth rate was then estimated as the slope of the fit with the highest Pearson’s \(r\) out of fits with the highest 80% of the estimated growth rates. If there was no single window of 6 consecutive points without missing values (due to cutoff, see above) in the growth curve, it was classified as representing no growth. These curves were assigned a growth rate of 0.

Yield (OD\(_{max}\)) was defined as the maximum OD over the duration of the whole experiment.

For plotting purposes, both OD\(_{max}\) and \(\mu_{max}\) were standardized relative to the strain carrying plasmid pBtetA-YFP in the absence of both tetracycline and arabinose.

Fluorescence data was corrected using the methods outlined in \cite{deJong2010}. Briefly, corrected fluorescence values YFP\(_c\) are calculated as

\[
YFP_c(t) = YFP(t) - s(OD_c(t)), \tag{2.2}
\]

where \(s\) is a correction function matching absorbance to autofluorescence values. This correction function is approximated by fitting a cubic smoothing spline to YFP as a function of OD\(_c\) for each time point of each replicate of the strain containing the control plasmid that carries no fluorophore. As a proxy of protein level during the experiment, the maximum corrected fluorescence over the whole time course (YFP\(_{max}\)) was used.
2.2. METHODS

Statistical analysis

The effects of induction of tetA on growth rate and yield were analyzed using a general additive model for location, scale and shape (Rigby and Stasinopoulos, 2005). In contrast to standard non-linear regression models, where the distribution of the response variable is assumed to be normal, these generalized models allow explicit modeling of the distribution of the response variable ($\mu_{\text{max}}$ or OD$_{\text{max}}$) as a function of the explanatory variables. This is necessary here, because our dataset features an excessive number of zero values, signifying no growth under the tested conditions.

The selection of the distribution of the response variable was based on comparing the Akaike information criterion (AIC) with a penalty $k = 2$ of several distributions fitted to the whole dataset (Appendix A, Fig. A.1). For $\mu_{\text{max}}$ the distribution used is a four parameter beta inflated distribution with location parameter $\mu$, scale parameter $\sigma$ and the two parameters $\nu$ and $\tau$ with the link functions logit, logit, log and log, respectively, for the four parameters. The parameters $\nu$ and $\tau$ model the probability density of the response variable at zero and one, respectively. For OD$_{\text{max}}$ a three parameter zero adjusted gamma distribution with the location parameter $\mu$, the scale parameter $\sigma$ and a parameter $\nu$ modeling the probability at zero was used. The link functions for parameters in this model are log, log and logit, respectively.

Both response variables were modeled using L-arabinose and tetracycline concentration as crossed factors and quadratic terms for both tetracycline and L-arabinose concentration. To correct for possible variation between the replicated assay plates, plate identity was included as a random effect. The models were fitted for each strain separately.

A linear mixed effects model was used to assess the effect of induction of tetA-YFP expression by L-arabinose and tetracycline. The response ($\text{YFP}_{\text{max}}$) was modeled with tetracycline and arabinose concentration as crossed fixed effects and plate as a random effect for each strain.
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separately. Again, to account for variations between replicated assay plates, plate identity was treated as a random effect.

For all fitted models significance was tested using restricted log-likelihood tests with a base significance level $\alpha = 0.05$. Comparison between means of individual groups were done using multiple Welch two sample $t$-tests with Bonferroni correction for multiple testing.

All statistical analysis was conducted in R (version 3.3.0, \cite{RCoreTeam2016}). General additive models were fitted using the \texttt{gamlss} function of the \texttt{GAMLSS} package (version 4.4.0, \cite{Stasinopoulos2007}). Linear mixed effects models were fitted using the \texttt{lmer} function of the \texttt{lme4} package (version 1.1-12, \cite{Bates2015}). Log-likelihood tests were performed using the \texttt{drop1} function of the R base package \texttt{stats}. All Plots were made in R using the \texttt{ggplot2} package (version 2.1.0, \cite{Wickham2009}).

2.3 Results and Discussion

**YFP fluorescence increases with increasing induction of \textit{tetA}-YFP**

For pTetA-YFP, L-arabinose induces expression of \textit{tetA}-YFP in a concentration dependent manner (significant effect of L-arabinose on fluorescence, $\chi^2(1) = 88.1$, $p < 0.001$; \textbf{Fig. 2.2b}). Expression is not completely shut down in the absence of L-arabinose as evidenced by significantly higher fluorescence in all but the highest tetracycline concentration (\textbf{Fig. 2.2b}, 0\% (w/v) L-arabinose) compared to the strain carrying plasmid pControl (\textbf{Fig. 2.2a}) (multiple $t$-test with Bonferroni correction, all $p < 0.001$).

Tetracycline induces the expression of \textit{tetA}-YFP from plasmid pTetRA-YFP. When \textit{tetA}-YFP is expressed from the tetracycline promoter on plasmid pTetRA-YFP, fluorescence/OD is larger at higher concentrations of tetracycline (significant effect of tetracycline concentration, $\chi^2(1) = 65.4$, $p < 0.001$). At tetracycline concentrations higher than
4µg/ml, no growth was observed. As expected L-arabinose does not influence the maximum fluorescence/OD of neither strain pControl nor strain pTetRA-YFP (no significant effect of L-arabinose concentration on fluorescence/OD: pControl: $\chi^2(1) = 2.72$, $p = 0.0990$, Fig. 2.2a; pTetRA-YFP: $\chi^2(1) = 0.119$, $p = 0.0730$, Fig. 2.2c).

Expression of tetA-YFP confers tetracycline resistance but is costly at high expression levels

tetA-YFP confers tetracycline resistance. At intermediate levels of tetA-YFP induction, growth rates and yield in the presence of tetracycline are comparable to those in the absence of tetracycline (Fig. 2.3) for both pBtetA-YFP and pTetRA-YFP. In case of pBtetA-YFP expression of tetA-YFP is induced by L-arabinose and growth in the presence of tetracycline but in absence of the inducer can be explained by incomplete repression of the promoter (see Fig. 2.3 and Fig. 2.2b). L-arabinose had no significant effect on growth rate or yield for the control strain ($\mu_{max}$: $\chi^2(1.00) = 0.822$, $p = 0.367$; OD$_{max}$: $\chi^2(1.00) = 0.00790$, $p = 0.929$; Appendix A, Fig. A.2). As expected, tetracycline inhibits the control strain with both growth rate and yield of the strain approximately zero for all tetracycline concentrations tested (Appendix A, Fig. A.2). Also for pTetRA-YFP, where tetA-YFP is expressed via the tetracycline promoter, L-arabinose had no effect on growth rate or yield ($\mu_{max}$: $\chi^2(1.00) = 0.0344$, $p = 0.853$; OD$_{max}$: $\chi^2(0.999) = 0.0153$, $p = 0.901$, Fig. 2.3).

Expression of tetA-YFP entails a reduction in both growth rate and yield. This effect can be observed at high levels of induction of tetA-YFP expression by L-arabinose, where growth rate and yield are significantly reduced (significant quadratic effect of L-arabinose on $\mu_{max}$, $\chi^2(1.00) = 7.71$, $p = 0.00550$ and on OD$_{max}$, $\chi^2(1.03) = 7.71$, $p = 0.00789$; Fig. 2.3). Growth rates and yield of the strain carrying plasmid pTetRA-YFP are also consistent with this observation, as high expression levels of tetA-YFP at high concentrations of tetracycline are correlated with a reduction in growth rate and yield (signifi-
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![Graph showing fluorescence/OD at different L-arabinose levels](image)

**Fig. 2.2** Maximum fluorescence/OD (arbitrary units) at different levels of L-arabinose (% (w/v)).

- **a)** Plasmid pControl, background fluorescence. No growth in the presence of tetracycline.
- **b)** Plasmid pBtetA-YFP. The effect of L-arabinose on fluorescence per OD is significant ($\chi^2(1) = 88.1, p < 0.001$). Expression of *tetA*-YFP is not completely off in the absence of L-arabinose, as evidenced by significantly higher fluorescence than the control strain in the absence of L-arabinose for all but the highest concentration of tetracycline (multiple t-test with Bonferroni correction, all $p < 0.001$).
- **c)** Plasmid pTetRA-YFP. The effect of L-arabinose on fluorescence per OD is not significant ($\chi^2(1) = 0.119, p = 0.730$) but tetracycline has a significant effect ($\chi^2(1) = 65.4, p < 0.001$) on fluorescence/OD. At tetracycline concentrations higher than 4µg/ml no growth was observed.

All plasmids are in strain BW27784 and were grown in M9 minimal media with 0.2% (w/v) glucose as the sole carbon source and 30µg/ml kanamycin to maintain the plasmid. Colors indicate different tetracycline concentrations in µg/ml. Plotted points and error bars represent the mean and s.e. of 19 - 20 replicates. a.u.: arbitrary units.
2.3. RESULTS AND DISCUSSION

a) growth rate

b) yield

Colors indicate different tetracycline concentrations (µg/ml). All plasmids are in strain BW27784 and were grown in M9 minimal media with 0.2% (w/v) glucose as the sole carbon source and 30µg/ml kanamycin to maintain the plasmid. Plotted points and error bars represent the mean and s.e. of 19 - 20 replicates.

Fig. 2.3 Effects of induction of *tetA*-YFP expression. a) Effects of L-arabinose on growth rate (*µ*max) for strains carrying plasmids pBtetA-YFP and pTetRA-YFP. Induction of *tetA*-YFP expression by L-arabinose or tetracycline (for pBtetA-YFP or pTetRA-YFP, respectively) significantly reduces growth rate (pBtetA-YFP: quadratic effect of L-arabinose on *µ*max, χ²(1.00) = 7.71, *p* = 0.00550; pTetRA-YFP: effect of tetracycline on *µ*max, χ²(1.03) = 7.03, *p* = 0.00843). b) Effects of L-arabinose on yield (ODmax) for strains carrying plasmids pBtetA-YFP or pTetRA-YFP. Induction of *tetA*-YFP expression by L-arabinose or tetracycline (for pBtetA-YFP or pTetRA-YFP, respectively) significantly reduces yield (pBtetA-YFP: quadratic effect of L-arabinose on ODmax, χ²(1.03) = 7.71, *p* = 0.00789; pTetRA-YFP: effect of tetracycline on ODmax, χ²(0.920) = 6.44, *p* = 0.00972).
cant effect of tetracycline on both $\mu_{\text{max}}$, $\chi^2(1.03) = 7.03$, $p = 0.00843$ and on $OD_{\text{max}}$, $\chi^2(0.920) = 6.44$, $p = 0.00972$; Fig. 2.3).

For pTetRA-YFP, expression levels at $2\mu g/ml$ of tetracycline were comparable to those for the strain with plasmid pBtetA-YFP with $10^{-4}\%$ (w/v) L-arabinose (Fig. 2.2), the highest expression level at which growth of the strain can still be observed (Fig. 2.3). This suggests that the costs related to expression are comparable between the constructs. In fact, only at $2\mu g/ml$ could strain pTetRA-YFP reach similar growth rates than in the absence of tetracycline. At $4\mu g/ml$ growth rates are reduced by about $80\%$ compared to the situation in the absence of tetracycline (Fig. 2.3). This indicates that the regulation of tetA-YFP expression from the tet promoter is not optimal at these concentrations. However, this might not be a feature of the tetRA regulon per se, but could stem from plasmid copy number effects amplifying expression levels at a given tetracycline concentration.

It has been previously shown that expression of tetA is costly both in terms of yield and growth rate in the absence of tetracycline when overexpressing tetA from a multi copy plasmid (Eckert and Beck, 1989). Our data confirms this finding. Nguyen et al. (1983) used competition assays and induction with anhydrotetracycline (a tetracycline analogue that is a strong inducer of tetA expression but a less potent antibiotic than tetracycline (Moyed et al., 1983)) to quantify the costs of expressing tetA in the absence of tetracycline by competing a strain with a plasmid containing tetRA to a strain not carrying the plasmid. They found significant competitive costs even at low induction of tetA expression. Competition assays in well-mixed environments are likely determined by the relative growth rates (Pfeiffer et al., 2001). In our experiment we only see reduction in growth rate at relatively high levels of tetA-YFP expression. This could indicate a different metabolic burden of tetA-YFP expression when expression of tetA is completely decoupled from the tetracycline concentration or the concentration of an analogous compound, e.g. because of energy expenditure due to activity of the tetracycline
2.3. Results and Discussion

Benefits of tetA-YFP expression depend on the concentration of tetracycline

Benefits of tetA-YFP expression, that is, the recovery of growth rate in the presence of tetracycline, are dependent on the tetracycline concentration and the level of expression (significant interaction of tetracycline concentration and induction by L-arabinose, $\chi^2(1.00) = 21.6, p < 0.001$). However, the same interaction is not significant for yield ($\chi^2(1.05) = 3.49, p = 0.0658$).

At a tetracycline concentration of 16$\mu$g/ml the maximum yield and growth rate is observed at intermediate levels of tetA-YFP expression (Fig. 2.3, 16 $\mu$g/ml tetracycline (red line) and Fig. 2.2b). This response to induction at high tetracycline concentrations suggests that there is an optimal expression level for this tetracycline concentration. This also shows that high levels of expression are also detrimental in the presence of tetracycline. This is apparent for both the inducible construct (pBtetA-YFP) as well as the tetRA regulon (pTetRA-YFP) suggesting that regulation of tetA did not only evolve to minimize costs in the absence of drug, but also to modulate costs and benefits in variable environments.

tetA-YFP expression is more costly in terms of yield than growth rate

Whether a given metabolic burden translates into a reduction in growth rate or yield (or other life-history traits) will depend on environmental conditions. Only in conditions were the specific metabolic burden entailed by the expression affects a limiting resource will the effect be visible (see also Chapter 1). We thus repeated our experiments in conditions where yield ($OD_{max}$) is limited by the available glucose (Bren et al., 2013).
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Fig. 2.4 Effects of induction of tetA-YFP expression under conditions where yield is glucose limited. a) Effects of L-arabinose on growth rate ($\mu_{max}$) for strains pBtetA-YFP and pTetRA-YFP. Effects of L-arabinose on growth rate of strain pBtetA-YFP are significant (quadratic effect of L-arabinose on $\mu_{max}$, $\chi^2(1.05) = 22.9$, $p < 0.001$). Strain pTetRA-YFP cannot grow at concentrations larger than 2µg/ml. b) Effects of L-arabinose on yield ($OD_{max}$) for strains pBtetA-YFP and pTetRA-YFP. Induction of tetA-YFP by L-arabinose reduces yield already at low concentrations (effect of L-arabinose on $OD_{max}$, $\chi^2(1.94)$, $p < 0.001$). Yield of strain pBtetRA is reduced already at a tetracycline concentration of 2µg/ml and no growth is observed at higher concentrations.

Colors indicate different tetracycline concentrations (µg/ml). Both strains were grown in M9 minimal media with 0.02% (w/v) glucose as the sole carbon source and 30µg/ml kanamycin to maintain the plasmid. Plotted points and error bars represent the mean and s.e. of 19 - 20 replicates.
While effects on growth rate were qualitatively the same in both tested glucose concentrations, effects on yield were already visible at lower arabinose concentrations for the strain pBtetA-YFP in the low glucose environment. We do not observe any growth of strain pTetRA-YFP at tetracycline concentrations larger than 2µg/ml in these conditions and yield is reduced substantially at this concentration (Fig. 2.4). This suggests that the costs of tetA-YFP expression are due to inefficiencies in carbon resource utilization that are more visible when these resources are limiting. This could be due to increased investment in protein production (Bragg and Wagner 2009), effects associated with the presence of the tetracycline efflux pump (e.g. disorganization of the inner membrane with associated loss of membrane potential (Eckert and Beck 1989)) or the specific or unspecific activity of the pump (similar to the case of the lactose symporter lacY, where activity is thought to be the main driver of costs (Eames and Kortemme 2012)).
2.4 Conclusion

Our results indicate that regulation of \textit{tetA} does not only contribute to the maintenance of tetracycline resistance in the absence of drug, but also minimizes the cost of resistance in the presence of drug in a concentration dependent manner. We show that costs are context dependent in terms of the available resources and that expression of \textit{tetA} is likely more costly in terms of yield than growth rate. This has potential consequences on the resulting selection of these traits. Depending on the population structure reductions in yield or growth rate can potentially have very different effects on allele frequencies over time (for an extensive discussion of how such trait effects possibly translate to effects on allele frequencies see \textsection{Chapter 1}).

Information on the quantitative relationship between antibiotic concentration and the cost-benefit trade-off of resistance gene expression is important to understand the fate of resistance genes in the environment and in clinical settings, possibly even enabling identification of conditions were resistance can be selected against.
References


2. COSTS & BENEFITS OF tetA


Chapter 3

Surviving sudden exposure to antibiotics: A “Catch-22”?  

Daniel C Angst, Daniel J Kiviet, Martin Ackermann, Sebastian Bonhoeffer
3. SUDDEN EXPOSURE TO ANTIBIOTICS

Abstract

Expression of resistance genes generally leads to a reduction in the rate at which bacteria form biomass or in the biomass yield. To reduce these costs in the absence of drug, some antibiotic resistance genes are regulated such that gene expression is only turned on in the presence of the antibiotic.

If expression of the resistance gene is completely suppressed in the absence of the drug this could potentially lead to a paradoxical situation in which the resistance gene can only be expressed when gene expression and protein synthesis is essentially impossible due to the presence of the drug, a so-called “catch-22” situation. This can happen if the upregulation of the gene and the production of the necessary proteins is too slow to counteract the effects of a suddenly appearing drug. This could especially be a problem for protein synthesis inhibitors, where the production of the resistance gene is directly inhibited by the drug.

Using both growth assays in batch and single-cell assays using time-lapse fluorescence microscopy we investigate whether there is such a “catch-22” situation in case of the tetA tetracycline efflux pump. TetA confers resistance to the protein synthesis inhibitor tetracycline and is directly regulated, through the repressor tetR, by tetracycline itself.

We observe a lag time on the population level when cells are exposed to tetracycline which is the result of growth of only a subpopulation of cells rather than an increase in single cell lag time of all cells. This shows that many genetically resistant cells are actually not phenotypically resistant, suggesting that inducible resistance in fact leads to a “catch-22” situation in this case.
3.1 Introduction

Expressing genes generally incurs metabolic costs that can lead to reductions in growth rate or yield. For genes that are non-essential (i.e. genes whose products are not required for growth in the current environment) gene regulation offers an effective way to reduce costs while maintaining the ability to respond to different environments. Prime examples are dedicated antibiotic resistance genes such as efflux pumps.

Most antibiotic resistance genes entail some sort of cost, often measured as a reduction of population growth rate or yield in the absence of the drug (see, for example, Chapter 2). Gene regulation can negate these costs in the absence of drug (Nguyen et al., 1989; Foucault et al., 2010) and can be tuned to maximize benefits across different drug concentrations (Chapter 2), similar to the case of the lac system in Escherichia coli (Dekel and Alon, 2005; Eames and Kortemme, 2012). However, complete suppression of gene expression could potentially lead to situations where cells that are genetically resistant cannot survive the sudden appearance of the drug, because upregulation and production of the necessary proteins might be too slow to counteract the effects of the drug.

Imagine a bacterial cell carrying a gene encoding a resistance protein. If the expression of this protein is induced by the drug itself, such that expression is completely shut off in the absence of drug, the cell will have no resistance protein before a sudden appearance of the drug (as is expected, for example at the onset of treatment) and have an essentially drug sensitive phenotype. Before the resistance mechanism can work the protein must be produced. Because the drug directly regulates the gene, this can only happen when the drug is in the cell and potentially also acts on its target. Especially in the case of drugs that inhibit protein synthesis (but conceivably also for other drugs) this could lead to a paradoxical situation where the resistance protein can only be synthesized when protein synthesis is essentially impossible. This could be referred to as a so called
“catch-22”, where contradictory rules make it impossible to resolve a situation.

We consider two, not mutually exclusive, explanations of how bacteria avoid this situation, enabling a population to continue to divide in the presence of a drug. i) *lagging population*: general purpose mechanisms (such as those controlled by the *mar* regulon ([Alekshun and Levy, 1999](#)) might enable cells to be metabolically active long enough to become phenotypically resistant or derepression, gene expression and translation might be quick enough to counteract any effects of the antibiotic before they are sufficiently large. ii) *heterogeneous population*: a subpopulation is already expressing the resistance gene and can resist the drug. This cell-to-cell heterogeneity could, for example, be due to stochastic gene expression or connected to the position in the cell cycle ([Ackermann, 2015](#)).

The two hypotheses described above imply essentially indistinguishable growth characteristics on the population level but different growth characteristics on the single cell level. On the population level we expect to see a prolonged lag phase in both cases (Fig. 3.1). For the lagging population this prolonged lag phase is the result of individual cells slowing down, or stopping, division until enough resistance protein is produced (Fig. 3.1 blue line). In the second case, the lag represents division of only a fraction of the population (Fig. 3.1 dashed orange line), while most of the population is drug susceptible and stops dividing (Fig. 3.1 dashed yellow line). On the population level, the growth characteristics can, depending on the length of the lag and the size of the subpopulation, be virtually indistinguishable (Fig. 3.1 blue and red line). Here we only consider bacteriostatic drugs, as most protein synthesis inhibitors, with the notable exception of macrolides, exhibit bacteriostatic activity. However, we expect growth curves to be similar to the ones presented in Fig. 3.1 for a bactericidal drug, with a slight reduction in population size of the heterogeneous population at the beginning.

On the single cell level the two hypotheses lead to different growth characteristics. For the lagging population we expect to see no di-
3.1. INTRODUCTION

Fig. 3.1  **Expected population growth after the sudden exposure to a bacteriostatic drug for a lagging and a heterogeneous resistant population.** The plot shows theoretical growth curves calculated using the model proposed by [Baranyi and Roberts (1994)](https://example.com). Even though the manner by which the population can survive the sudden appearance of the drug is different in the two cases, the growth curves (and ) can be essentially identical (as shown here), depending on the subpopulation size and the lag time.

- : Population size (log-scale) over time for an untreated population.
- : Population size (log-scale) over time of the lagging population. Every cell can, after a prolonged lag phase where the resistance protein is slowly produced, become phenotypically resistant and start dividing.
- : Population size (log-scale) over time of a heterogeneous population. A subpopulation of cells can divide immediately, because it already expressed the resistance gene before exposure to drug.   is the sum of the dividing subpopulation ( ) and the non dividing subpopulation ( ).

The lagging population and the dividing subpopulation have the same maximum growth rate and the same total initial and maximal population size. The untreated and the dividing subpopulation show no lag. The initial size of the dividing subpopulation is 1/50 of the initial population size. For more details on the model, see [Appendix B](https://example.com) [Section B.1](https://example.com).
visions for a time period, corresponding to the lag phase on the population level, before cells start to divide again. For the heterogeneous population we expect that a minority of cells (i.e. the dividing subpopulation, Fig. 3.1, orange dashed line) start dividing with no, or a much shorter, lag time than in the previous case because they are already expressing the resistance gene. The rest of the population is not phenotypically resistant and does not divide in the presence of the drug (Fig. 3.1, yellow dashed line).

We used the tetracycline resistance operon tetRA to investigate how cells survive the sudden appearance of an antibiotic. Tetracycline (Tc) is a bacteriostatic antibiotic that inhibits protein synthesis (Thaker et al., 2010). Tc enters the cell through outer membrane proteins and then by passive diffusion through the inner membrane (Schnappinger and Hillen, 1996). Inside the cell, Tc reversibly binds to the 30S subunit of the ribosome preventing the binding of tRNA to the ribosome, thus stalling translation. 

\textit{tetA} encodes for a tetracycline-H\textsuperscript{+} antiporter that pumps tetracycline through the inner membrane into the periplasm. The expression of \textit{tetA} is regulated via the repressor TetR. In the absence of tetracycline, TetR binds to two operators between the divergently oriented \textit{tetR} and \textit{tetA} genes, tightly repressing expression of both genes (Hillen et al., 1983; Chopra and Roberts, 2001). When cells are exposed to the drug, tetracycline enters the cell and binds to TetR releasing the repressor allowing for expression of both \textit{tetR} and \textit{tetA}.

We analyzed both population level effects and effects on single cells using growth experiments in batch and time-lapse fluorescence microscopy to investigate the response of resistant bacteria to the sudden exposure to tetracycline on the population and single cell level.
3.2 Methods

Strain and Media

We use *Escherichia coli* BW27784 ([Khlebnikov et al., 2001](#)) carrying plasmid pTetRA-YFP in all experiments. Plasmid pTetRA-YFP is a synthetic plasmid carrying a tetRA cassette where tetA is translationally fused to a YFP fluorophore. The tetRA sequence was obtained from the NCBI Nucleotide database (GenBank Accession Number: AF162223.1, see also [Chalmers et al., 2000](#)). YFP (GenBank Accession Number: AJW76798.1) is fused to tetA with a 18bp Glycine-Serine linker replacing both the stop codon of tetA and the start codon of the YFP gene. The backbone is pJ247, with a high copy pUC origin of replication. The plasmid was synthesized and sequence verified by DNA2.0 (Newark, California, USA). Full sequences of the plasmid is provided in [Appendix A, Section A.3](#).

Strains were grown at 37°C in M9 minimal media with 0.02% (w/v) or 0.2% glucose for the single cell and batch culture experiments, respectively, or on LB agar (1.5% agar). Solid media and liquid media for the batch culture experiment were supplemented with kanamycin at a concentration of 30 µg/ml to maintain the plasmid. In the single cell experiments, 30µg/ml kanamycin was only added at the end of the experiment to test whether the cells still contained the plasmid. For single cell experiments the media was further supplemented with 0.01% Tween 20 (Fluka 93773) to prevent cell clumping. Tetracycline stock solutions were prepared in 95% ethanol and diluted into growth medium at the concentrations indicated.

Measuring lag time in batch culture

To measure lag time, fresh overnight cultures were diluted 1:10 in media containing 0, 2 or 4µg/ml tetracycline and incubated at 37°C for about 4 hours (approximately the time needed for cultures to reach mid-exponential growth) before they were diluted 1:10 into fresh media again containing 0, 2, 4 µg/ml tetracycline. Cultures
were diluted such that all combinations of tetracycline concentrations before and after dilution were obtained. After dilution the cultures were incubated at 37°C for 12 hours.

The experiment was performed in 6 biological replicates in a 96-well black polystyrene clear-bottom plate (Costar 3603). The wells on the edge of the plate were filled with growth media, but not used for the experiment. We also included 6 blank wells as sterile controls and to use for background correction. The plate was incubated in a Tecan Infinite F200 Spectrophotometer (Tecan, Männedorf, Switzerland). Both before and after the dilution, optical density at 595nm (OD) of the cultures was measured every 5 minutes. The plate was shaken for 30 seconds and allowed to settle for 5 seconds before each read.

Background corrected $OD_c$ was calculated by subtracting the mean of all blank wells at the beginning of the experiment from the measured OD values. $OD_c$ values below an arbitrary cutoff of 0.001 were discarded. To calculate lag time, we first calculated the maximum specific growth rate $\mu_{max}$ by fitting linear models (i.e. $\log_2(OD_c) = \mu_{max}t + b$) to a sliding window of 48 log$_2$-transformed $OD_c$ reads (approximately 4 hours). Maximum specific growth rate was then estimated as the slope of the fit with the highest Pearson’s $r$ out of fits with the highest 80% of the estimated growth rates. To estimate lag time we extrapolated exponential growth back in time to the point where it intersects the minimal observed OD after dilution ($OD_{min}$):

$$\text{lag time} = \frac{\log_2(OD_{min}) - b}{\mu_{max}} - t_{OD_{min}}$$

(3.1)

where $b$ is the intercept of the linear model used to calculate $\mu_{max}$. This value represents the time difference between a culture that immediately starts growing exponentially with the measured growth rate and the observed culture. Longer lag times indicate that some time passed until the population reached its maximum specific growth rate $\mu_{max}$.
3.2. METHODS

Time-lapse fluorescence microscopy and image analysis

We used a microfluidic device ([Wang et al., 2010](#)) that allows the observation of many individual bacterial cells over an extended period of time. The microfluidic device consists of many small growth channels (width $1 \mu m$, length $13 \mu m$, height $0.9 \mu m$) that open up into a large flow channel (width $200 \mu m$, height $23 \mu m$) where fresh media is continuously provided. The media is pumped through the flow channel at $0.5 ml/h$ with a syringe pump. The needle of the syringe was connected to the media inlet of the device with $40 cm$ of thick tubing (Tygon S-54-2L, $0.76 mm$ ID x $2.29 mm$ OD) and $40 cm$ of thin tubing (PTFE, $0.3 mm$ ID x $0.76 mm$ OD). To load the device, $1 ml$ of a fresh overnight culture was centrifuged for $60 s$ at $14400 rpm$, decanted and resuspended in approximately $20 \mu l$ of supernatant and injected into the device through the media outlet. Cells were forced into the growth channels by slowly moving an air bubble through the flow channel. Once enough cells entered the growth channels, media flow was started. The device was placed on the automated stage of an Olympus BX81 fluorescence microscope and multiple fields of views were observed over time. Phase contrast and YFP fluorescence (EYFP filter U-N49003 (Chroma, Bellows Falls, Vermont, USA), Excitation 490-510nm, Emission range 520-550nm) images were acquired every 6 minutes for every position. The microfluidic device and the microscope were temperature controlled at $37^\circ C$ with a cube-and-box incubation system (Life Imaging Services, Reinach, Switzerland).

After about 10 hours of growth on media without tetracycline, of which at least the last $2 h$ hours were recorded, the media was switched to media containing tetracycline at a concentration of $2$ or $4 \mu g/ml$. As a control, a switch to media without tetracycline was also performed. The switching was performed by disconnecting the tubing from the syringe and attaching it to another syringe containing the new media. Two independent experiments were conducted on different days. The first experiment only included the control treatment and the switch
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to 4µg/ml tetracycline.

The acquired images were analyzed using the Fiji distribution [Schindelin et al., 2012] of ImageJ (version 1.51d, Schneider et al., 2012). Phase and fluorescence images were merged, cropped and registered before analysis. For each channel in the image one focal cell was selected and marked using the “Lasso/Blow Tool”. Each cell was then observed through all acquired frames and division events were recorded manually. In addition, cells were marked in the frame just before the media switch and at the end of the experiment or in the last frame that the cell was in the channel. For these regions mean pixel value of the fluorescence image of the marked region was extracted. The fluorescence values were background corrected by subtracting the mean of the mean pixel value of three empty regions in three different channels in the frame where the fluorescence measurement was taken. Single cell lag times were estimated as the time to the next division after the switch to media containing tetracycline.

**Statistical Analysis**

For the statistical analysis we assume that the individual cells can be treated as independent samples. Division and fluorescence data obtained from the single cell experiments were analyzed using Mann-Whitney U tests comparing division times and fluorescence before and after the switch to tetracycline as well as between surviving and non-surviving cells. This non-parametric test was chosen because of low sample sizes in some groups. When two or more comparisons were made p-values were adjusted using the Bonferroni method.

Difference in lag times between groups were analyzed using a one-way ANOVA for each concentration of tetracycline before dilution, testing for effects of the tetracycline concentration after dilution on lag time. Differences between individual groups were assessed using Tukey-Kramer HSD post-hoc tests (Kramer, 1956). To test for difference from zero of the groups t-tests with Bonferroni correction for multiple testing were used.
All statistical analysis was conducted in R (version 3.3.0, R Core Team, 2016). All Plots were made in R using the ggplot2 package (version 2.1.0, Wickham, 2009).

3.3 Results and Discussion

Substantial lag in population growth after sudden exposure to tetracycline

On the population level our proposed hypotheses of how resistant cells manage to survive the sudden onset of tetracycline (Tc) both predict a longer lag phase, either because individual cells need time until they can divide again or because only a subpopulation can divide (see Fig. 3.1). Consistent with this prediction and previous results (Lenski et al., 1994), the resistant strain has a prolonged lag time when diluted into media containing Tc from exponential growth in the absence of drug. The lag time is longer the higher the Tc concentration is (ANOVA, $F_{2,15} = 23.562$, $p < 0.001$, Fig. 3.2a). As expected, when diluted into media containing no drug we observe no lag (One sample t-test with Bonferroni correction for multiple testing: $t_5 = -0.176$, $p = 1$). All growth curves are shown in Appendix B, Fig. B.1.

We also measured lag times when cells were grown in the presence of Tc before the dilution. For both concentrations tested, we see no effect of the Tc concentration after dilution on lag time (ANOVA: 2µg/ml Tc: $F_{2,15} = 0.821$, $p = 0.459$; 4µg/ml Tc: $F_{2,14} = 0.490$, $p = 0.623$; Fig. 3.2b and c). When strains were diluted into the same concentration of Tc lag times were not significantly different from zero (One sample t-test with Bonferroni correction for multiple testing: 2µg/ml Tc: $t_5 = 1.48$, $p = 1$, Fig. 3.2b; 4µg/ml Tc: $t_4 = 1.61$, $p = 1$, Fig. 3.2c). In the case of growth in 4µg/ml Tc before dilution, dilution into different concentrations of Tc lead to non-zero lag times (0µg/ml Tc: $t_5 = 5.09$, $p = 0.0341$, 2µg/ml Tc: $t_5 = 6.33$, $p = 0.013$; Fig. 3.2c). However, the same is not true for growth in 2µg/ml Tc.
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![Graph](image)

**Fig. 3.2** Lag time after dilution into media with or without tetracycline.

a) Growth in media without tetracycline (Tc) before dilution. Concentration of Tc after dilution has a significant effect on lag time (ANOVA, $F_{2,15} = 23.562$, $p < 0.001$). Lag times when diluted into media containing Tc are larger than zero (One sample t-tests with Bonferroni correction for multiple testing: $2 \mu g/ml$ Tc: $t_5 = 5.98$, $p = 0.00187$, $4 \mu g/ml$ Tc: $t_5 = 7.37$, $p = 0.00651$). b) Growth in media with $2 \mu g/ml$ Tc before dilution. No significant effects of Tc concentration (ANOVA, $F_{2,15} = 0.821$, $p = 0.459$) and none of the treatments lead to lag times significantly larger than zero (all $p \geq 0.05$). c) Growth in media with $4 \mu g/ml$ Tc before dilution. No significant effects of Tc concentration on lag time (ANOVA, $F_{2,14} = 0.490$, $p = 0.623$). Lag times when diluted into Tc concentrations lower than before the switch were significantly larger than 0 ($0 \mu g/ml$ Tc: $t_5 = 6.33$, $p = 0.013$, $2 \mu g/ml$ Tc: $t_5 = 6.33$, $p = 0.013$).

Cells were grown in M9 with 0.2% glucose and 30$\mu g/ml$ Kanamycin to maintain the plasmid. The media was supplemented with Tc at the concentrations indicated. Thick and thin lines indicates mean ± the standard error of the mean. Line and symbols above the data indicate results of a post-hoc Tukey test comparing all groups. **∗∗∗**: $p < 0.001$; **∗∗**: $p < 0.01$; **∗**: $p < 0.05$; n.s.: not significant ($p \geq 0.05$).
before dilution \((0\mu g/ml: t_5 = 3.17, p = 0.223, 4\mu g/ml \text{ Tc}: t_5 = 1.48, p = 1)\). Still, the absence of a prolonged lag phase when diluted into the same concentration of Tc suggests that the increase in lag time, observed when diluting into higher concentration of Tc, is connected to the expression of \(tetA-YFP\). These cells could still need to produce more TetA to divide under these conditions. The non-zero lag times when diluting into lower concentrations of tetracycline could be a result of costs of the expression of \(tetA-YFP\) or the presence of the efflux pump (see also \textit{Chapter 2}).

Many resistant cells do not divide after the sudden exposure to tetracycline

In order to differentiate between a homogeneous lagging population and a heterogeneous population, where only a subset of the cells can divide in the presence of Tc, we analyzed single cell growth of cells in microfluidic device before and after a sudden exposure to Tc. We extracted cell division events and fluorescence before and after the switch from microscopy images acquired at 6 minute intervals (Fig. 3.3). We performed two experiments and as the results of the two experiments are qualitatively similar we mostly discuss one of the two experiments. Data from the other experiment is included in \textit{Appendix B} and differences in the outcomes between the two experiments are discussed in the main text.

Many of the genetically resistant cells fail to divide after a switch to tetracycline (Fig. 3.3). When the media is suddenly switched to 4\(\mu g/ml\) tetracycline only 20 out of 49 cells (41\%) that divided before the switch manage to divide again after the switch (Fig. 3.4). In the other experiment, this ratio was even smaller with only 31\% (9 out of 29) of cells dividing after the switch (Fig. B.6 and Fig. B.7).

This observation is consistent with the hypothesis, that only a sub-population can divide after the sudden exposure to tetracycline. As outlined above and in Fig. 3.1 this could explain the increased lag time observed in batch culture experiments (Fig. 3.2).
3. Sudden Exposure to Antibiotics

**Fig. 3.3** Division events and fluorescence before and after the media switch. Every horizontal line is an observed cell (observed cells that did not divide over the course of the experiment are not shown). The color of the line before and after the switch (indicated by the gray background) represents measured fluorescence (arbitrary units) just before the switch and at the end of the experiment, respectively. All cells were grown in a microfluidic device in M9 with 0.02% glucose and 0.01% Tween 20. After the switch the media was supplemented with the indicated concentration of tetracycline.

- **a)** Control treatment: switch to media containing no tetracycline.
- **b)** Switch to 2µg/ml tetracycline.
- **c)** Switch to 4µg/ml tetracycline.

- •: cell division; ◊: cell loss, i.e. the observed cell is washed out of the channel; ×: cell lysis.
Fig. 3.4  *Ratio of cells still dividing after sudden exposure to different concentrations of tetracycline.* Even though the cells are genetically resistant, many fail to divide after a sudden switch to media containing tetracycline. Numbers in bars are dividing cells before and dividing cells after the switch.

All cells were grown in a microfluidic device in M9 with 0.02% glucose and 0.01% Tween 20. After the switch the media was supplemented with the indicated concentration of tetracycline.
No evidence for prolonged single cell lag time after sudden exposure to tetracycline

Even though we found support for the hypothesis that only a sub-population is able to divide after the sudden exposure to Tc, it is still conceivable that these cells also show a lag phase before initiating division after the switch. We quantify single cell lag time as the time from the switch until the first division after the switch. In both experiments we could not detect any difference between the control treatment and the switch to different concentrations of tetracycline (Fig. 3.5 and Fig. B.8).

This indicates that the lag time observed on the population level (Fig. 3.2) is not due to individual cells not dividing after dilution into media containing tetracycline. Together with the direct observation of a dividing subpopulation this lends additional support for the hypothesis that only a subpopulation can continue to divide after the sudden exposure to Tc, and that this subpopulation is able to do so without a substantial lag phase.

We investigated two possible mechanisms that could explain why only some cells manage to divide. First, the dividing subpopulation might already be expressing \textit{tetA} and maintaining a small amount of TetA and can thus counter the inflow of Tc when the drug appears. Second, the position in the cell cycle of individual cells might influence their ability to divide after exposure.

No correlation between survival and fluorescence before the switch

In our experiments we used a Tc efflux pump that is translationally fused to a YFP fluorophore (see Section 3.2). This allows us to quantify the amount of TetA-YFP in individual cells. As expected fluorescence is significantly higher in cells exposed to Tc after the switch than before the switch (see Appendix B, Fig. B.2 and Fig. B.3). To check whether the cells that manage to divide after the switch were
3.3. RESULTS AND DISCUSSION

Fig. 3.5  **Time to next division after media switch.** We find no evidence for a prolonged single cell lag time, as measured as the time to next division after the switch to media containing the indicated concentration of tetracycline (two sided Mann-Whitney $U$ test with bonferroni correction for multiple comparison, $n_{0\mu g/ml\ Tc} = 49$, $n_{2\mu g/ml\ Tc} = 19$, $n_{4\mu g/ml\ Tc} = 20$, all $p > 0.05$).

Thick and thin lines indicates the non parametric population estimate and the upper and lower bounds of the 95% confidence interval for groups with 5 or more samples. Line and symbols above the data indicate results of a two sided Mann-Whitney $U$ test comparing the groups with Bonferroni correction for multiple comparison. n.s.: not significant ($p \geq 0.05$).
already expressing *tetA*-YFP, we compared the fluorescence before the switch of cells that managed to divide after the switch to those that did not (Fig. 3.6).

We found no evidence that cells that express *tetA*-YFP before the switch are more likely to divide after the switch to tetracycline (2µg/ml Tc: Mann-Whitney $U = 134.5$, $n_{dividing} = 19$, $n_{not\ dividing} = 16$, $p = 0.554$; 4µg/ml Tc: Mann-Whitney $U = 293$, $n_{dividing} = 20$, $n_{not\ dividing} = 29$, $p = 0.957$). The same effect is significant for the control treatment (0µg/ml Tc: Mann-Whitney $U = 30$, $n_{dividing} = 49$, $n_{not\ dividing} = 7$, $p < 0.001$) which would indicate that cells that continue to divide have a lower fluorescence. However the effect size is small (95% CI: [-2.254, -0.886]) compared to fluorescence of induced cells (see Appendix B, Fig. B.2). In the other experiment only two cells did not divide after the switch (Appendix B, Fig. B.3) so we cannot draw any conclusions. It is however not unconceivable that cells that have a higher expression are at danger of losing viability, as this is consistent with substantial costs caused by the expression of *tetA* [Eckert and Beck 1989, Nguyen et al. 1989](#) see also Chapter 2.

As a measure of the costs of expressing *tetA*-YFP, we also quantified interdivision time before and after the switch to tetracycline (see Appendix B, Section B.4) but found no consistent results. We found indication for a longer interdivision time after the switch to Tc only for the 2µg/ml Tc treatment (2µg/ml Tc: Mann-Whitney $U = 146$, $n_{before} = 10$, $n_{after} = 18$, $p = 0.00766$; 4µg/ml Tc: Mann-Whitney $U = 111$, $n_{before} = 12$, $n_{after} = 13$, $p = 0.0734$, Fig. B.4b and c). However, the same effect was also significant for the control treatment in the other experiment (Mann-Whitney $U = 1143$, $n_{before} = 31$, $n_{after} = 47$, $p < 0.001$, Fig. B.4a) but not the first experiment (Mann-Whitney $U = 328.5$, $n_{before} = 22$, $n_{after} = 23$, $p = 0.0882$, Fig. B.5a). So unlike in Chapter 2, where we investigated the costs and benefits of *tetA*-YFP expression and found a marked decrease in growth rate at 4µg/ml Tc, we cannot see this on the single cell level in terms of interdivision time.
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Fig. 3.6 Fluorescence before switch and survival. We found no evidence that cells that express tetA-YFP before the switch are more likely to divide after the switch to tetracycline (Tc) (2µg/ml Tc: Mann-Whitney U = 134.5, n\textit{dividing} = 19, n\textit{not dividing} = 16, p = 0.554; 4µg/ml Tc: Mann-Whitney U = 293, n\textit{dividing} = 20, n\textit{not dividing} = 29, p = 0.957). The same test is significant for the control treatment (0µg/ml Tc: Mann-Whitney U = 30, n\textit{dividing} = 49, n\textit{not dividing} = 7, p < 0.001), but difference is small (95% CI: [-2.254, -0.886]) compared to fluorescence of induced cells (see Appendix B, Fig. B.2). Thick and thin lines indicate the non parametric population estimate and the upper and lower bounds of the 95% confidence interval for groups with 5 or more samples. Line and symbols above the data indicate results of a two sided Mann-Whitney U test comparing the groups. ***: p < 0.001, **: p < 0.01, *: p < 0.05, n.s.: not significant (p ≥ 0.05).
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Cell cycle position and survival

As another possible explanation for the dividing subpopulation we looked at the position in the cell cycle of cells that manage to divide after the switch. Protein production rates in *E. coli* fluctuate over the time period of about one cell cycle (Rosenfeld *et al.*, 2005; Ackermann, 2015) and the position in the bacterial cell cycle of an individual cell can influence its ability to persist when exposed to stressors (Mathis and Ackermann, 2016), including antibiotics in *Mycobacterium smegmatis* (Aldridge *et al.*, 2012). The position in the cell cycle could thus also be a factor that determines whether a genetically resistant cell can continue to divide after exposure to tetracycline. We estimated cell cycle position as the time since last division at the switch to tetracycline (Fig. 3.7).

We found no evidence for an effect of cell cycle position on division after the switch to media containing Tc (2 µg/ml Tc: Mann-Whitney *U* = 137.5, *n* dividing = 19, *n* not dividing = 16, *p* = 0.641; 4 µg/ml Tc: Mann-Whitney *U* = 375, *n* dividing = 20, *n* not dividing = 29, *p* = 0.0845; Fig. 3.7b and c). We found a significant effect in the control treatment (Mann-Whitney *U* = 68, *n* dividing = 49, *n* not dividing = 7, *p* = 0.0105; Fig. 3.7a) indicating that cells further in their cell cycle had a harder time to divide after the media switch. One potential explanation for this pattern is that these cells were dividing more slowly before the switch and then stopped dividing. However, sample size is, as expected, low (*n* not dividing = 7). In the other experiment the sample size is too low to draw conclusions (Mann-Whitney *U* = 10.5, *n* dividing = 22, *n* not dividing = 2, *p* = 0.249). It is thus doubtful whether this significance is meaningful.
3.3. RESULTS AND DISCUSSION

Fig. 3.7 Time since last division at the time of the switch to tetracycline. The time since the last division (min) at the time of the media switch is a proxy for cell cycle position. There is no evidence for an effect after switch to tetracycline (2µg/ml Tc: Mann-Whitney $U = 137.5$, $n_{\text{dividing}} = 19$, $n_{\text{not dividing}} = 16$, $p = 0.641$; 4µg/ml Tc: Mann-Whitney $U = 375$, $n_{\text{dividing}} = 20$, $n_{\text{not dividing}} = 29$, $p = 0.0845$). We found a significant difference in the time since last division for the control treatment (Mann-Whitney $U = 68$, $n_{\text{dividing}} = 49$, $n_{\text{not dividing}} = 7$, $p = 0.0105$), this could be due to these cells dividing slower already before the switch. $*$: $p < 0.05$, n.s.: not significant ($p \geq 0.05$).
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3.4 Conclusion

Our data confirms the hypothesis that many genetically resistant cells are in fact not phenotypically resistant to tetracycline and cannot, at least in the observed time frame, divide after the sudden exposure to tetracycline. This is consistent with the idea that cells with the inducible tetracycline resistance operon *tetRA* are in a “catch-22” situation.

The fact that the proportion of cells that manage to divide after the media switch seems to be concentration dependent ([Fig. 3.4](#)) would be consistent with cells stochastically producing some TetA even in the absence of tetracycline, thus leading to a distribution of number of tetracycline efflux pumps in the cells. We cannot confirm this with our fluorescence measurements, however, it is possible that our methods lack the sensitivity to measure low enough levels of *tetA*-YFP expression. We found no evidence for an effect of position in cell cycle on the probability of dividing after the switch.

The finding that a large fraction of genetically resistant cells are not phenotypically resistant in a situation where the concentration of the drug rises sharply – as is expected during treatment – could be interesting to combat resistance. It has been shown that tetracycline resistant strains are more susceptible to lipophilic chelating agents such as fusaric acid ([Bochner et al., 1980](#)). Combination therapy with such a compound and tetracycline could possibly be used to treat tetracycline resistant pathogens. Tetracycline would reduce the population size considerably, even though the population is genetically resistant, and the second compound could specifically inhibit the remaining, phenotypically resistant, cells.
References


3. SUDDEN EXPOSURE TO ANTIBIOTICS


Chapter 4

The cost of antibiotic resistance depends on evolutionary history in *Escherichia coli*

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Abstract

Background
The persistence of antibiotic resistance depends on the fitness effects of resistance elements in the absence of antibiotics. Recent work shows that the fitness effect of a given resistance mutation is influenced by other resistance mutations on the same genome. However, resistant bacteria acquire additional beneficial mutations during evolution in the absence of antibiotics that do not alter resistance directly but may modify the fitness effects of new resistance mutations.

Results
We experimentally evolved rifampicin-resistant and sensitive *Escherichia coli* in a drug-free environment, before measuring the effects of new resistance elements on fitness in antibiotic-free conditions. Streptomycin-resistance mutations had small fitness effects in rifampicin-resistant genotypes that had adapted to antibiotic-free growth medium, compared to the same genotypes without adaptation. We observed a similar effect when resistance was encoded by a different mechanism and carried on a plasmid. Antibiotic-sensitive bacteria that adapted to the same conditions showed the same pattern for some resistance elements but not others.

Conclusions
Epistatic variation of costs of resistance can result from evolution in the absence of antibiotics, as well as the presence of other resistance mutations.
4.1 Background

The persistence of antibiotic-resistant bacteria depends on how resistance, in the form of chromosomal mutations or horizontally acquired elements such as plasmids, affects fitness relative to antibiotic-sensitive genotypes in the absence of antibiotics (Andersson and Hughes, 2010; Andersson and Levin, 1999). Recent work shows that the fitness effects of resistance mutations often vary depending on the presence of other resistance mutations on the same genome (Rozen et al., 2007; Salverda et al., 2011; Trindade et al., 2009; Ward et al., 2009; Weinreich et al., 2005). However, resistance evolution will often be accompanied by the fixation of additional mutations that do not confer resistance but increase fitness in the present environment, either because resistant bacteria evolve in heterogeneous hosts or natural environments where the optimal genotype changes over time, or because the cost of resistance causes selection for compensatory mutations (e.g. Cohan et al., 1994; Kugelberg et al., 2005; Levin et al., 2000; Paulander et al., 2007; Reynolds, 2000; Schrag et al., 1997). Any impact of adaptation in the absence of antibiotics on the subsequent cost of additional resistance mutations therefore potentially modulates costs of resistance in natural populations of pathogens.

Epistatic interactions have been observed among different resistance elements (Rozen et al., 2007; Salverda et al., 2011; Trindade et al., 2009; Silva et al., 2011; Weinreich et al., 2006), and among different beneficial mutations during adaptation to novel environments (Chou et al., 2011; Khan et al., 2011; Kvitek and Sherlock, 2011; Tenali-lon et al., 2012). However, interactions between the two types of mutations in terms of how they influence fitness in the absence of antibiotics are less clear. In this paper we ask whether adaptation of antibiotic-resistant and sensitive bacteria to the same antibiotic-free environment alters the fitness effects of additional resistance elements that confer resistance against other antibiotics. To test for this possibility, we experimentally evolved rifampicin-resistant (Rif\textsuperscript{R}) and rifampicin-sensitive (Rif\textsuperscript{S}) \textit{E. coli} in liquid growth medium in
the laboratory, before inserting mutations that conferred resistance to streptomycin (Str\textsuperscript{R}), shown schematically in Fig. 4.1. Str\textsuperscript{R} mutations on \textit{rpsL} change the structure of ribosomal protein S12 and interfere with target binding in the presence of streptomycin (Alekshun and Levy, 2007; Maisnier-Patin \textit{et al.}, 2007; Ozaki \textit{et al.}, 1969). To determine whether observed effects were specific to \textit{rpsL} mutations, we carried out parallel experiments with a plasmid that confers resistance to sulfonamides and streptomycin (Sul\textsuperscript{R} + Str\textsuperscript{R}). The plasmid we used (RSF1010) has a broad host range and encodes resistance to streptomycin by enzymatic modification (Schölz \textit{et al.}, 1989). Therefore our experiment incorporated multiple distinct antibiotic-resistance mechanisms. We measured the fitness effects of resistance elements by pairwise competition assays, allowing us to quantify the effects of additional resistance elements for Rif\textsuperscript{R} and Rif\textsuperscript{S} bacteria both before and after adaptation. In several cases we found that the cost of a new resistance element was smaller in genotypes that had adapted to our experimental environment compared with the same genotypes without adaptation, although Rif\textsuperscript{R} and Rif\textsuperscript{S} bacteria showed different patterns for some resistance elements.

### 4.2 Materials & Methods

#### Bacterial strains & growth conditions

In all experiments we used \textit{E. coli} MG1655 grown at 37\textdegree{}C. To isolate mutants resistant to either rifampicin or streptomycin (Rif\textsuperscript{R} or Str\textsuperscript{R}), we plated independent cultures of the wild type onto LB agar supplemented with 50 mg/L rifampicin (Hall, 2013) or 25 mg/L streptomycin. After 24 h incubation at 37\textdegree{}C we picked individual colonies, restreaked to purify genotypes and confirm resistance, before growth for 2 h in liquid LB and storage at −80\textdegree{}C in 25\%v:v glycerol. Rifampicin-resistance mutations were identified by sequencing the central resistance-determining region of rpoB as described previously (Hall, 2013); streptomycin-resistance muta-
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Fig. 4.1 Construction of different genotypes and associated resistance phenotypes. (1) The wild type and three Rif\textsuperscript{R} genotypes evolved for approximately 200 generations in liquid LB. (2) Additional resistance elements were inserted in the form of Str\textsuperscript{R} mutations on \textit{rpsL} (K43N or K88R) or a Sul\textsuperscript{R} + Str\textsuperscript{R} plasmid (RSF1010). Fitness scores for all genotypes are given in [Appendix C, Table C.1].
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tions were identified by sequencing part of rpsL using primers fwd 5’-ATGATGGCGGGATCGTTG-3’ and rev 5’-CTTCCAGTTCAGATTACC-3’ (Trindade *et al.* 2009). All three of the RifR mutations (D516G, S512F, I572S) and both StrR mutations (K43N and K88R) have been previously associated with resistance to these antibiotics in *E. coli* (Trindade *et al.* 2009; Reynolds 2000; Garibyan *et al.* 2003). The SulR + StrR plasmid RSF1010 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). We constructed double-resistant genotypes, with two resistance mutations or one resistance mutation plus the plasmid, by transduction or transformation as described below.

**Experimental evolution in the absence of antibiotics**

We initiated selection lines with each of the three RifR genotypes (D516G, I572S, S512F) and three replicate lines with the wild type (Fig. 4.1). Each selection line was grown in 100µl liquid LB medium and diluted 1000-fold into fresh medium approximately every 12 h, as described previously (Hall 2013). After 20 transfers, approximately 200 generations, we plated every population onto LB agar and isolated a single colony from each, which we then grew for 2 h in liquid LB and stored at −80°C. We checked for reversion to rifampicin-sensitivity (RifS) in the evolved clones derived from resistant genotypes by plating on LB agar supplemented with rifampicin, but observed none. This confirmed that the colony isolates used in the present study remained RifR, although it does not exclude the possibility of revertants at low frequency in evolved populations. This procedure yielded nine different genotypes in addition to the wild type (left-hand side of Fig. 4.1): three RifR genotypes that had not adapted to LB, three RifR-evolved genotypes that had adapted to LB (RifR-evolved), and three RifS genotypes that had adapted to LB (RifS-evolved).
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Addition of streptomycin resistance mutation and plasmid

We used P1 transduction to insert either K43N or K88R into each of the nine genotypes described above, plus three independent replicates of the wild type. We followed Thomason et al. (2007) with few modifications. Briefly, a lysate of each donor strain was prepared by growth with phage P1 at low multiplicity of infection until lysis was visible, then transduced to the relevant recipient genotype by growth in LB for 20 min, followed by addition of sodium citrate, further incubation for 1 h, plating on LB agar plus streptomycin and sodium citrate, and restreaking three times before isolating a single colony at random and storing at $-80^\circ$C. All constructed genotypes were verified by resequencing rpsL. Transductions were done in a single temporal block; for one genotype (I572S + K88R) a different Str$^R$ mutation was acquired during the isolation procedure; for D516G + K43N, no colonies were obtained after repeated attempts; these are excluded from further analysis.

Plasmid RSF1010 is a natural, nontransmissible, broad-host range plasmid of the IncQ incompatibility group conferring resistance to sulfonamide and streptomycin (Scholz et al., 1989). We extracted the plasmid from cells grown in LB using the PureYield Plasmid Miniprep Kit (Promega). We then transformed the nine isolated genotypes and three replicates of the wild type using TSS Transformation (Chung et al., 1989). Briefly, strains were grown in LB to OD600 0.3 and chilled on ice for 10 min before adding an equal volume of ice cold 2x TSS (TSS is LB with 10% w:v PEG8000, 5% v:v DMSO, 50 mM MgSO4 at pH 6.5) and incubation on ice for another 30 minutes. We then added 1 ml of competent cells to 1 µl (~100 ng) of the plasmid prep and incubated on ice for one hour. After incubation at 37°C for one hour to allow expression of the resistance genes, we plated cells on LB agar supplemented with 30 mg/L streptomycin. For each transformation a single colony was isolated at random, grown for 2 h in liquid LB and stored at $-80^\circ$C.
The fitness effects of transduced mutations and the plasmid were reproducible in independently constructed replicates of the wild type: fitness did not differ significantly among different isolates for any of the resistance elements in our experiment (K43N: $F_{2,4} = 3.00, P = 0.16$; K88R: $F_{1,4} = 0.05, P = 0.83$; plasmid: $F_{2,6} = 1.33, P = 0.33$). To check that the plasmid was not lost during competition assays, we plated all competitions that included a plasmid-carrying genotype on tetrazolium arabinose (TA) plates, where streptomycin-resistant genotypes form white colonies as described below, and on LB agar supplemented with 30 mg/L streptomycin, on which plasmid-carrying genotypes can grow but the wild type cannot. We observed approximately the same number of colonies on streptomycin plates as white colonies on TA plates (paired t-test: $t_{35} = 1.49, P = 0.14$), indicating that the plasmid was maintained throughout the competition.

**Fitness measurements**

We measured the competitive fitness of each genotype against a marked strain: *E. coli* K12 MG1655 Δara, which is otherwise isogenic to the wild type and forms red colonies on TA agar (tryptone 1%, yeast extract 0.1%, NaCl 0.5%, L(+)arabinose 1%, TTC 0.005%). For each competition, we grew independent cultures of both competitors overnight in liquid LB, before mixing them 1:1 v:v and diluting 1000-fold into fresh LB media. We estimated the frequency of each competitor by plating the culture on TA agar before and after two growth cycles using the same protocol as during experimental evolution. We then calculated relative fitness, $w$, as $1 + s$, where $s$ is the selection coefficient $s = \ln(R_{final}/R_{initial})/t$, where $R_{final}$ and $R_{initial}$ are the ratios of the competing genotypes at the beginning and end of the assay and $t$ is assay duration in generations (Trindade *et al.*, 2009; Gullberg *et al.*, 2011; Lenski *et al.*, 1991); in batch culture $t$ can be approximated as $\log_2(N_{final}/N_{initial})$, where $N$ is total population size. We discounted each score by the cost of the Δara marker (Trindade *et al.*, 2009), which was not significantly
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different from zero (−0.025 on average; P = 0.06). To calculate the fitness effects of StrR mutations or the plasmid in a given genetic background, we took the difference in fitness (Δw) between the same strain with and without K43N, K88R or the plasmid.

RifR, RifR-evolved and RifS-evolved genotypes lacking either StrR mutations or the plasmid were each assayed in three different blocks: once after the evolution experiment to test for adaptation (Hall, 2013), once alongside genotypes with K43N or K88R, and once alongside genotypes carrying the plasmid. The correlation between fitness scores relative to the wild type measured for these genotypes in different blocks was high (r² = 0.91, 0.94, 0.92), and there was no block × genotype interaction (F₁₆,₄₆ = 1.61, P = 0.10), indicating that fitness values of different genotypes relative to each other were repeatable across blocks of assays. In each block, competitions were replicated three times independently, and nine times for the wild type.

**Testing for chaperone (DnaK and GroEL) overproduction**

The best-described molecular mechanism for buffering against the fitness effects of deleterious mutations is the overproduction of molecular chaperones (Fares et al., 2002b; Kerner et al., 2005; Tokuriki and Tawfik, 2009), enzymes that assist in correct protein folding (Rutherford, 2003). To determine whether variation of costs of resistance among genotypes in our experiment could be explained by variation in chaperone production, we measured levels of the two key chaperones DnaK and GroEL (Maisnier-Patin et al., 2005) using western blots. Cells were harvested by centrifugation from mid-exponential cultures, resuspended in 1x SDS-PAGE sample buffer and boiled for 10 minutes. SDS-PAGE and immunoblotting with mouse-monoclonal anti-DnaK and anti-GroEL antibodies (Enzo Life Sciences) was carried out according to standard procedures. ImmunStar Western C Substrate Kit (BioRad) was used for detection.
in conjunction with horseradish peroxidase-conjugated anti-Mouse IgG secondary antibody (Amersham). Blots were imaged using the ChemiDoc XRS + CCD System (BioRad) and analyzed using ImageJ 1.46 (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2012.).

**Statistical Analysis**

We tested for adaptation to LB by a paired $t$-test using the average fitness of each genotype before and after experimental evolution. We tested for variation of the response to selection among genotypes by analysis of variance including genotype and assay block as factors. To test whether adaptation to LB altered the average fitness effects of additional resistance elements (Str$^R$ mutations or the plasmid) we conducted paired $t$-tests, taking the average fitness effect ($\Delta w$) of each resistance element on each genetic background before and after evolution, measured in the same block of competition assays. We analyzed variation of fitness effects among genotypes that had or had not adapted to LB by analyses of variance with genotype as a factor. Additionally, we tested pairwise epistatic interactions of Rif$^R$ mutations with Str$^R$ mutations or the plasmid in genotypes that had not adapted to LB. We did this using the multiplicative model described by Trindade *et al.* (2009) and results are shown in Appendix C, Table C.2. Finally, to test for differences in levels of chaperones DnaK and GroEL we used pairwise $t$-tests with non-pooled standard deviation, correcting for multiple comparisons using the Bonferroni method.

**4.3 Results**

**Adaptation in antibiotic-free conditions**

Antibiotic-resistant (Rif$^R$) bacteria and the antibiotic-sensitive wild type increased in fitness over approximately 200 generations of evolution in LB growth medium (paired $t$-test: $t_5 = 6.20, P = 0.002$;
Fig. 4.2). The change in fitness after experimental evolution varied among different evolved genotypes ($F_{5,31} = 52.99, P < 0.0001$). Among RifR genotypes, the response to selection was relatively large for genotypes derived from D516G and S512F, which had lower fitness than the wild type at the start of the experiment, compared with I572S that had a fitness advantage. After experimental evolution, RifR and RifS genotypes had similar levels of fitness on average (Welch’s $t$-test: $t_{2.35} = 0.40, P = 0.72$; Fig. 4.2). Thus, despite initial variation, resistant and sensitive genotypes adapted to experimental conditions and converged upon similar levels of competitive fitness, as shown previously for a wider range of rpoB mutants including the three in this study (Hall, 2013).

**Fitness effects of streptomycine resistance mutations**

The fitness effects of StrR mutations were smaller on average in genotypes that had adapted to LB compared to the same genotypes without adaptation (paired $t$-test: $t_8 = 4.83, P = 0.001$; Fig. 4.3a,b). Across all genetic backgrounds, K43N was more costly on average than K88R (mean fitness effect ± s.d. K43N: $-0.11 ± 0.07$; K88R: $-0.01 ± 0.04$; Fig. 4.3a,b).

For K43N, all of the RifR-evolved and RifS-evolved genotypes we tested paid a smaller cost of resistance than the same genotypes without evolution in LB (Fig. 4.3a). Among genotypes that had not adapted to LB, the cost was similar in S512F and the wild type, but comparatively small for I572S. This represents antagonistic pairwise epistasis between I572S and K43N: the cost of having both mutations was smaller than expected from their independent effects on the wild type (Appendix C, Table C.2). Among evolved genotypes, the cost of K43N also varied depending on genetic background ($F_{4,10} = 12.84, P < 0.001$), but in every case it was less costly than without evolution in LB.

When streptomycin resistance was due to K88R, the fitness cost was smaller after adaptation to LB for both of the RifR genotypes
Fig. 4.2 Adaptation of resistant and sensitive bacteria. Three Rif\textsuperscript{R} genotypes, denoted on the x-axis by their amino acid changes, and three replicates of the wild type (WT) were evolved for approximately 200 generations in antibiotic-free LB medium as described previously (Hall, 2013). White and hatched bars show scores before and after evolution respectively. Each bar shows average ± s.e. competitive fitness relative to the wild type from nine independent assays, conducted in three temporal blocks; for the wild type before evolution standard error is shown for 27 assays.

we tested, although this only represented statistically significant variation for S512F (Fig. 4.3b). The fitness effect of K88R varied among genotypes that had not adapted to LB ($F_{2,9} = 4.67, P = 0.04$), being costly on average in Rif\textsuperscript{R} genotypes but not the wild type. K88R was also approximately neutral for two out of three evolved wild type (Rif\textsuperscript{S}) genotypes, and for the remaining one it had a small
positive fitness effect. Consequently, adaptation to LB had no effect on average for the cost of K88R in Rif$^S$ genotypes ($F_{1,3} = 0.31, P = 0.62$). By contrast, K88R was costly to Rif$^R$ genotypes before, but not after experimental evolution in LB.

In summary, for one Str$^R$ mutation (K43N) the fitness cost was consistently lower for evolved genotypes than the same genotypes without adaptation to LB. A similar effect was observed for the other Str$^R$ mutation on Rif$^R$ genetic backgrounds, but not for Rif$^S$ genotypes, where K88R was approximately neutral both before and after evolution in LB on average. Thus, the fitness effects of Str$^R$ mutations varied epistatically depending on both adaptation in the absence of antibiotics and on the presence of other resistance mutations.
Fitness effects on an antibiotic-resistance plasmid

Insertion of the plasmid RSF1010, conferring resistance to sulfonamides and streptomycin, had a marginal cost on average (mean fitness effect ± s.d. = −0.03 ± 0.05), but this varied considerably among genotypes depending on adaptation to LB and RifR mutation (Figure 3c). There was no difference on average between the cost of RSF1010 in genotypes that had evolved in LB and those that had not (paired t-test: $t_5 = 1.29, P = 0.25$), although in some cases the fitness effect of the plasmid was clearly lower in evolved genotypes. Specifically, RSF1010 was less costly for genotypes with RifR mutations D516G or I572S after they had evolved in LB (Fig. 4.3c), but there was no difference for S512F or any of the RifS evolved genotypes. This variation was driven by a comparatively large fitness cost in two of the unevolved RifR genotypes, reflecting negative pairwise epistasis between the plasmid and D516G and I572S (Table C.2). Thus, the plasmid was costly to D516G and I572S before but not after evolution in antibiotic-free LB; other genotypes paid a comparatively small cost of carrying the plasmid.

Buffering of fitness effects is not due to overexpression of chaperones

The phenotypic effects of deleterious mutations can be buffered by overproduction of molecular chaperones, of which GroEL and DnaK are the two most important and best studied (Tokuriki and Tawfik, 2009; Maisnier-Patin et al., 2005). However, we found no upregulation of either chaperone in evolved genotypes (pairwise t-tests: all $P$ values > 0.075; Appendix C, Fig. C.1).

4.4 Discussion

We measured the fitness effects of three different antibiotic-resistance elements (two StrR mutations and a SulR + StrR plasmid) in RifR and RifR genotypes that had or had not adapted to antibiotic-free growth.
medium. In cases where additional resistance elements were costly to genotypes that had not evolved in LB, the cost was consistently smaller after adaptation to our experimental environment. For one of the Str$^R$ mutations this pattern was consistent across Rif$^R$ and Rif$^S$ genotypes, suggesting that the relatively small cost of new resistance mutations after adaptation in the absence of antibiotics is not specific to resistant bacteria. For the other Str$^R$ mutation and the plasmid, the cost was greatest in Rif$^R$ genotypes that had not adapted to LB, and evolved genotypes showed a small cost or even a benefit of resistance by comparison. These results show that evolution in the absence of antibiotics can alter the fitness effects of new resistance elements, but this pattern depends on the identity of the new mutation and on the presence of other resistance mutations.

Consistent with previous studies [Rozen et al., 2007; Trindade et al., 2009; Ward et al., 2009; Weinreich et al., 2006; Hall and MacLean, 2011], we found pairwise epistatic interactions among resistance elements on the same genetic background: out of seven multiple-resistant genotypes with a Rif$^R$ mutation and either an Str$^R$ mutation or the plasmid, three showed significant deviations from a multiplicative model of fitness (Appendix C, Table C.2). Moreover, our finding that evolution in the absence of antibiotics alters the fitness effects of additional resistance elements suggests that epistatic variation of costs of resistance can also be caused by beneficial mutations that fix in the absence of antibiotics.

To our knowledge, the only well-characterized mechanism that actively buffers against deleterious mutations is over-expression of molecular chaperones [Maisnier-Patin et al., 2005; Fares et al., 2002a; Tokuriki et al., 2007]. This did not explain our findings: the two main chaperones, DnaK and GroEL, were not overproduced in evolved genotypes. This is perhaps unsurprising, given that selection for chaperone-mediated buffering is expected to depend on intense genetic drift (Gros and Tenaillon, 2009) or high mutation rates (de Visser et al., 2003; Wagner, 2005), which is supported by experiments with viruses (Sanjuán et al., 2007). In the absence of
direct selection for a buffering mechanism, the impact of adaptation on the cost of Str$^R$ mutations and the plasmid is probably an indirect effect of mutations that were under positive selection for their effects on growth in LB. By analogy, $rpoB$ mutations that fix under selection for rifampicin resistance have wide-ranging effects on the bacterial phenotype, owing to direct effects on the function of RNA polymerase and pleiotropic effects on the expression of other genes (Campbell et al., 2001; Derewacz et al., 2013; Perkins and Nicholson, 2008). This is associated with altered growth phenotypes in unselected environments (Perkins and Nicholson, 2008; Trindade et al., 2012) and epistatic interactions with other resistance mutations (Trindade et al., 2009; Ward et al., 2009). Although we lack a physiological understanding of adaptation to LB, it is known to alter growth phenotypes in unselected environments to a similar degree as $rpoB$ mutations in this experimental system (Hall, 2013). This is consistent with beneficial mutations fixed in LB having indirect phenotypic effects that are unrelated to improved growth in LB but may generate epistatic interactions.

Our experiments included a limited set of resistance elements (three different types, including chromosomal mutations and a plasmid). Therefore we do not draw any general conclusions about how adaptation will influence the cost of new resistance elements. However, our results do show that adaptation in the absence of antibiotics can cause epistatic variation of fitness costs. In support, a recent study demonstrated variation of the fitness effects of rifampicin-resistance mutations among antibiotic-sensitive E. coli genotypes (Rodríguez-Verdugo et al., 2013). This is consistent with epistatic variation of fitness costs due to mutations at loci unrelated to drug resistance. We also stress that with only one selection line for each Rif$^R$ genotype, variation in the outcomes of evolution among evolved Rif$^R$ genotypes does not necessarily reflect an effect of starting genotype. Stochastic processes during adaptation, including the random appearance and loss of beneficial mutations (Travisano et al., 1995), will also contribute to variation among individual selection lines. Therefore,
while we found differences on average between Rif$^R$ and Rif$^S$ evolved genotypes, and between evolved and nonevolved genotypes, we did not test whether the effect of adaptation varies among genotypes with different rifampicin-resistance mutations.

Directions for further work include identifying the physiological mechanisms by which adaptation to antibiotic-free conditions can alter the costs of new resistance elements, and how general such effects are. For example, expression profiling of resistant and sensitive genotypes that have and have not been experimentally evolved could be used to find cellular functions that are altered by resistance mutations and adaptation, both independently and in combination. To determine whether adaptation has a general effect on the cost of additional resistance mutations would require experiments with other model organisms, antibiotics and growth media. In particular, testing whether compensatory mutations that ameliorate the deleterious effects of one resistance mutation (Cohan et al., 1994; Levin et al., 2000; Paulander et al., 2007; Reynolds, 2000; Schrag et al., 1997) can buffer against the effects of resistance elements at other loci would be relevant to the broader question of how selection acts on antibiotic resistance across genetic backgrounds.

4.5 Conclusions

We observed epistatic variation of the fitness costs associated with antibiotic resistance, stemming from interactions between different resistance elements as observed previously (Rozen et al., 2007; Trindade et al., 2009; Ward et al., 2009) and from interactions between resistance elements and beneficial mutations that fixed during adaptation to drug-free conditions. This is potentially relevant for the cost of resistance in real populations of pathogenic bacteria. In chronic infections, such as those caused by *Pseudomonas aeruginosa* in cystic fibrosis patients, bacteria fix multiple mutations during a single infection, many of which are unrelated to resistance evolution (Ciofu et al., 2010; Huse et al., 2010; Smith et al., 2006). Other pathogens such as
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*Mycobacterium tuberculosis*, which often carry antibiotic resistance elements, also fix mutations that are beneficial in the absence of antibiotics (Comas et al., 2012). If such mutations influence the cost of subsequent resistance mutations, as they did in our experiment, then a better understanding of epistasis between resistance elements and other types of mutations will be important for predicting the likelihood that multi-drug resistant bacteria will persist following different types of treatment.

**Authors’ contributions**

DCA and ARH designed and performed the experiments, analyzed data, wrote the paper and approved the final manuscript.

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References


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General Discussion

Costs and benefits are the main drivers of the evolution and the persistence of resistance genes. Exactly how costs influence evolutionary processes is influenced by the biotic and abiotic environment of the bacteria on at least two levels. First, how physiological effects, for example the use of additional resources for a resistance mechanism, translates to a trait effect like a reduction in yield depends on the availability of these resources. Second, whether this reduction translates into a selective effects will depend on e.g. the spatial structure of the environment (Chapter 1).

Gene regulation adds another layer to this. The environmental factors that induce the expression of resistance mechanisms can reduce the costs that these mechanism incur in the absence of drug (Chapter 2). In Chapter 2 we present evidence that gene regulation also minimizes costs in the presence of drug in the \textit{tetRA} system. A thorough quantitative understanding of the gene regulation function can possibly allow us to identify regimes where regulation of the resistance gene is suboptimal or highlight interventions that would disturb the optimal regulation. These regimes could then potentially be exploited to maximize the biological costs of resistance, making the spread and persistence of the resistance gene less likely.

The nature of the gene regulation function can also tell us about the environment in which the regulation has evolved and what the drivers shaping the gene regulation function are (Kalisky \textit{et al.}, 2007).
One way to investigate this would be to experimentally adapt an inducible resistance mechanism to different regimes of fluctuating drug concentrations. The resulting adaptations could shed light on the environment to which the inducible resistance gene is best adapted and also could give us further information about possible trade-offs of inducible resistance.

We investigated one trade-off of inducible resistance in Chapter 3. Here the fact that in the absence of tetracycline most of the genetically resistant cells are phenotypically susceptible and the population is rescued by a phenotypically resistant subpopulation can be seen as a trade-off. For such a mechanism to be selected for, the benefits of not expressing the resistance gene in the absence of the drug must outweigh the costs of loosing a large part of the population when the drug suddenly appears. This depends on the frequency of such events in the environment where the regulation has evolved. However, it is not clear whether the mechanism we studied actually evolved in a setting where the increase of the tetracycline concentration is as sudden as in our experiments. The tetracycline regulation system is one of the most sensitive regulation systems known with only nanomolar concentrations of tetracycline needed for induction (Chopra and Roberts, 2001). If the concentration of tetracycline rises slowly it is possible that cells can express and translate tetA before the drug concentration reaches inhibitory levels. It is thus possible that when concentrations of tetracycline are rising less quickly more cells will survive. The microfluidic setup used in Chapter 3 will allow us to test this hypothesis by changing the time-scale at which the switch to tetracycline happens.

Our finding that only a subpopulation of a genetically resistant population actually survives the treatment with tetracycline and the indication that the size of this subpopulation is smaller the higher the concentration of the drug is, has potential implications on treatment with tetracycline. Treating with the highest amount of drug possible and making sure that the concentration of the drug at the site of infection rises sharply should ensure that only few of the genetically
resistant bacteria survive. However, this approach still suffers from the problem of aggressive chemotherapy that Read et al. (2011) outline: aggressive chemotherapy maximizes the selective advantage of resistant bacteria directly by enhancing the relative advantage of resistant cells and indirectly by killing sensitive cells that compete for common resources. This strategy could therefore enhance the spread of this resistance, but could still be useful by reducing the population size of the resistant infection to levels where the immune system could handle the infection (Read et al., 2011). This might be a valid approach when the prevalence of resistance is already high.

We could not find the mechanism how the surviving subpopulation (that is genetically identical to the rest of the population) survives the sudden onset of the drug. Understanding this mechanism could potentially allow the design of a treatment regime where these surviving cells would be specifically targeted by another compound. Approaches like this, i.e. combining drugs to which resistance is widespread with compounds that specifically target resistant bacteria, ideally through a mechanism directly related to the specific resistance, could allow to prolong the effective lifetime of existing antibiotics. One such mechanism could be the increased susceptibility of tetracycline resistant strains against lipophilic chelating agents such as fusaric acid (Bochner et al., 1980).

In this thesis I have presented work highlighting the need to take a closer look at costs and benefits of antibiotic resistance. To be able to predict the evolution and spread of antibiotic resistance we need to have a quantitative understanding of the effects – both on a physiological level and concerning selection – of resistance and how they vary with the environment. Insight into what environmental factors influence costs and benefits of resistance could be used to optimize treatment regimes or the management of environmental hot spots for selection of resistance (such as for example waste water treatment plants) such that selection for resistance is slowed or even reversed.
References


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Appendices
Appendix A

Biological Costs and Benefits of \textit{tetA}
A.1 Selection of response variable distribution for statistical models

![Histogram of $\mu_{\text{max}}$](image1)

![Histogram of $\text{OD}_{\text{max}}$](image2)

**Fig. A.1** Histograms of response variables $\mu_{\text{max}}$ and $\text{OD}_{\text{max}}$. Lines show fits of four distributions to the whole dataset. NO: Normal distribution, BEINF: four parameter Beta inflated distribution, BEINF0: three parameter Beta zero inflated distribution, ZAGA: three parameter zero adjusted Gamma distribution. AIC values reported are for a penalty $k = 2$. 
A.2 Growth rate and yield of strain pControl

Fig. A.2  Growth rate and yield of strain BW27784 with plasmid pControl at different levels of L-arabinose (% (w/v)). As expected the addition of L-arabinose has no significant effect on both growth rate ($\chi^2(1.01) = 0.822$, $p = 0.367$) and yield ($\chi^2(1.00) = 0.0079$, $p = 0.929$). a) maximum relative growth rate $\mu_{\text{max}}$ (min$^{-1}$), b) yield (OD$_{\text{max}}$)

The plasmid is in strain BW27784 grown in M9 minimal media with 0.2% (w/v) glucose as the sole carbon source and 30$\mu$/ml kanamycin to maintain the plasmid. Colors indicate different tetracycline concentrations in $\mu$/ml. Plotted points and error bars represent the mean and s.e. of 19 - 20 replicates.
A.3 Sequences

pTetRA-YFP

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FEATURES

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A.3. SEQUENCES

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//
# APPENDIX A. COSTS & BENEFITS OF \( \text{tetA} \)

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**ORGANISM**

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**FEATURES**

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APPENDIX A. COSTS & BENEFITS OF \textit{tetA}

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APPENDIX A. COSTS & BENEFITS OF \textit{tetA} \\

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APPENDIX A. COSTS & BENEFITS OF \textit{tetA}

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\end{verbatim}
Appendix B

Surviving sudden exposure to antibiotics: A “Catch-22”? 
Appendix B. Sudden Exposure to Antibiotics

B.1 Theoretical Growth curves

To illustrate the two mechanisms of how a population of inducibly resistant bacteria can continue to grow after the sudden exposure to drug we generated two theoretical growth curves. We used the growth model developed by Baranyi and Roberts (1994) to calculate population size over time. The explicit form of the model is:

\[ y(t) = y_0 + \mu_{\text{max}} t + \frac{1}{\mu_{\text{max}}} \ln(e^{-\nu t} + e^{-h_0} - e^{-\nu t - h_0}) - \frac{1}{m} \ln \left( 1 + \frac{e^{m\mu_{\text{max}} t + \frac{1}{\mu_{\text{max}}} \ln(e^{-\nu t + e^{-h_0} - e^{-\nu t - h_0}) - 1}{e^{m(y_{\text{max}} - y_0)}} \right) \]

\[ \text{Eq. B.1} \]

where

- \( y(t) \) is the natural logarithm of population size over time,
- \( y_0 \) is the natural logarithm of the initial population size,
- \( y_{\text{max}} \) is the natural logarithm of the maximal population size,
- \( \mu_{\text{max}} \) is the maximum specific growth rate,
- \( m \) is a parameter characterizing the transition out of the exponential phase,
- \( \nu \) is a parameter characterizing the transition to the exponential phase and
- \( h_0 \) is a parameter characterizing the initial physiological state of the cells.

As suggested by Baranyi and Roberts (1994) \( m \) is set to 1 (i.e. assuming logistic potential growth) and \( \nu \) is set to be equal to \( \mu_{\text{max}} \).
In this model lag time is defined as \( \frac{h_0}{\mu_{max}} \). To generate Fig. 3.1 we calculated \( y(t) \) using the parameters shown in Table B.1. The growth curve of the total population size of the heterogeneous population (Fig. 3.1, red line) is the sum of the population size of the two subpopulation (orange dashed line (dividing subpopulation) and yellow dashed line (non dividing subpopulation)). With this parameter set the lagging population and the heterogeneous population are virtually indistinguishable on the population level.

**Table B.1** Parameter used to calculate theoretical growth curves. Growth curves where calculated using the model by Baranyi and Roberts (1994) (Equation B.1).

<table>
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<tr>
<th></th>
<th>( y_0 )</th>
<th>( y_{max} )</th>
<th>( \mu_{max} )</th>
<th>( h_0 )</th>
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<td>( \ln(1) )</td>
<td>( \ln(10^9) )</td>
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<td>0</td>
</tr>
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<td>lagging population</td>
<td>( \ln(1) )</td>
<td>( \ln(10^9) )</td>
<td>1</td>
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<td>heterogeneous population</td>
<td>( \ln(1) )</td>
<td>( \ln(10^9) )</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>growing subpopulation</td>
<td>( \ln(0.02) )</td>
<td>( \ln(10^9) )</td>
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<td>non growing subpopulation</td>
<td>constant ( y(t) = \ln(0.98) )</td>
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</table>
B.2 Growth curves from batch growth experiment

**Fig. B.1** Growth curves of BW27784 (pTetRA-YFP) when diluted into media containing tetracycline. The plot shows log$_2$-transformed, background corrected OD over time for strain BW27784 with plasmid pTetRA-YFP. After 260 minutes of growth the cultures were diluted into fresh media containing the same or a different concentration of tetracycline (indicated by the gray background).

- **a - c)** growth in 0 µg/ml tetracycline before dilution. Dilution into 0 (**a**), 2 (**b**) and 4 µg/ml (**c**) tetracycline.
- **d - f)** growth in 2 µg/ml tetracycline before dilution. Dilution into 0 (**d**), 2 (**e**) and 4 µg/ml (**f**) tetracycline.
- **g - i)** growth in 4 µg/ml tetracycline before dilution. Dilution into 0 (**g**), 2 (**h**) and 4 µg/ml (**i**) tetracycline.

Cells were grown in M9 with 0.2 % glucose and 30 µg/ml kanamycin to maintain the plasmid.
B.3 Induction of tetA-YFP by tetracycline

Fig. B.2  Tetracycline induces expression of tetA-YFP. Fluorescence is significantly higher after the switch to media containing tetracycline (Tc) for cells that divide both before and after the media switch (2µg/ml Tc: Mann-Whitney $U = 314$, $n_{before} = 19$, $n_{after} = 19$, $p < 0.001$; 4µg/ml Tc: Mann-Whitney $U = 326$, $n_{before} = 20$, $n_{after} = 20$, $p < 0.001$). The same effect is also significant for the control switch (0 µg/ml Tc: Mann-Whitney $U = 1574$, $n_{before} = 49$, $n_{after} = 49$, $p = 0.00513$), however in this case the estimated effect small compared to the treatments with Tc (0µg Tc: 95% CI $[5.08 \times 10^{-5}, 0.526]$; 2µg/ml Tc: 95% CI $[20.106, 63.702]$; 4µg/ml Tc: 95% CI $[0.752, 89.379]$).

Thick and thin lines indicates the non parametric population estimate and the upper and lower bounds of the 95% confidence interval for groups with 5 or more samples. Line and symbols above the data indicate results of a two sided Mann-Whitney $U$ test comparing the groups. ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, n.s.: not significant ($p \geq 0.05$).
**Fig. B.3** Tetracycline induces expression of *tetA-YFP*: Data from replicate experiment. Fluorescence is significantly higher after the switch to media containing tetracycline (Tc) for cells that divide both before and after the media switch (Mann-Whitney $U = 81$, $n_{before} = 9$, $n_{after} = 9$, $p < 0.001$). The same effect is not significant for the control switch (0 µg/ml Tc: Mann-Whitney $U = 208.5$, $n_{before} = 22$, $n_{after} = 22$, $p = 0.416$).

Thick and thin lines indicates the non parametric population estimate and the upper and lower bounds of the 95% confidence interval for groups with 5 or more samples. Line and symbols above the data indicate results of a two sided Mann-Whitney $U$ test comparing the groups. ***: $p < 0.001$, n.s.: not significant ($p \geq 0.05$).
B.4 Interdivision times in single-cell experiments

**Fig. B.4** Interdivision times before and after the switch to tetracycline. Mean interdivision time is longer after the media switch for the control treatment and for the switch to 2μg/ml tetracycline (Tc) (0μg/ml Tc: Mann-Whitney \( U = 1143, n_{before} = 31, n_{after} = 47, p < 0.001; 2\mu g/ml Tc: Mann-Whitney \( U = 146, n_{before} = 10, n_{after} = 18, p = 0.00766 \)). The same effect is not significant for the switch to 4μg/ml Tc (Mann-Whitney \( U = 111, n_{before} = 12, n_{after} = 13, p = 0.0734 \)). Only cells that divide at least once both before and after the switch are included.

Thick and thin lines indicates the non parametric population estimate and the upper and lower bounds of the 95% confidence interval for groups with 5 or more samples. Line and symbols above the data indicate results of a two sided Mann-Whitney \( U \) test comparing the groups. \(* * *: p < 0.001, **: p < 0.01, *: p < 0.05, \) n.s.: not significant \((p \geq 0.05)\).
**Fig. B.5 Interdivision times before and after the switch to tetracycline (Tc): Data from replicate experiment.** Mean interdivision time are not significantly different after the media switch (0µg/ml Tc: Mann-Whitney $U = 284.5$, $n_{before} = 21$, $n_{after} = 22$, $p = 0.197$; 4µg/ml Tc: Mann-Whitney $U = 42$, $n_{before} = 8$, $n_{after} = 8$, $p = 0.328$). Only cells that divide at least once both before and after the switch are included.

Thick and thin lines indicates the non parametric population estimate and the upper and lower bounds of the 95% confidence interval for groups with 5 or more samples. Line and symbols above the data indicate results of a two sided Mann-Whitney $U$ test comparing the groups. n.s.: not significant.
**B.5 Additional data plots from replicate experiment**

![Graph showing cell division and fluorescence over time](image)

**Fig. B.6 Division events and fluorescence before and after the media switch: Data from replicate experiment.** Every horizontal line is an observed cell (observed cells that did not divide over the course of the experiment are not shown). The color of the line before and after the switch (indicated by the gray background) represents measured fluorescence (arbitrary units) just before the switch and at the end of the experiment, respectively.

All cells were grown in a microfluidic device in M9 with 0.02% glucose, 0.01% Tween 20. After the switch the media was supplemented with the indicated concentration of tetracycline.

- **a)** Switch to media containing no tetracycline.
- **b)** Switch to 2µg/ml tetracycline.
- •: cell division; ◊: cell loss, i.e. the observed cell is washed out of the channel; × cell lysis
Fig. B.7  **Ratio of cells still dividing after a sudden switch to different concentrations of tetracycline: Data from replicate experiment.** Even tough the cells are genetically resistant, many fail to divide after a sudden switch to media containing tetracycline. Numbers in bars are dividing cells before and dividing cells after a sudden switch to tetracycline.

All cells were grown in a microfluidic device in M9 with 0.02% glucose, 0.01% Tween 20. After the switch the media was supplemented with the indicated concentration of tetracycline.
Fig. B.8  **Time to next division after media switch: Data from replicate experiment.** We find no evidence for a prolonged single cell lag time, as measured as the time to next division after the switch to tetracycline.

Thick and thin lines indicates the non parametric population estimate and the upper and lower bounds of the 95% confidence interval for groups with 5 or more samples. Line and symbols above the data indicate results of a two sided Mann-Whitney \( U \) test comparing the groups (Mann-Whitney \( U = 74.5, n_{0\mu g/mlTc} = 22, n_{4\mu g/mlTc} = 9, p = 0.294 \)). n.s.: not significant \( (p \geq 0.05) \).
**Fig. B.9**  Fluorescence before switch and survival: Data from replicate experiment. We found no evidence that cells that express tetA-YFP before the switch are more likely to divide after the switch to tetracycline (Tc) (0µg/ml Tc: Mann-Whitney $U = 24.5$, $n_{\text{dividing}} = 22$, $n_{\text{not dividing}} = 2$, $p = 0.831$; 4µg/ml Tc: Mann-Whitney $U = 90.5$, $n_{\text{dividing}} = 9$, $n_{\text{not dividing}} = 20$, $p = 1$).

Thick and thin lines indicates the non parametric population estimate and the upper and lower bounds of the 95% confidence interval for groups with 5 or more samples. Line and symbols above the data indicate results of a two sided Mann-Whitney $U$ test comparing the groups. n.s.: not significant ($p \geq 0.05$).
Fig. B.10  **Time since last division at the time of the switch to tetracycline: Data from replicate experiment.** No difference in time since last division at the time of the switch, a proxy for cell cycle position, for surviving versus non surviving cells for all treatments (0µg/ml tetracycline: Mann-Whitney $U = 10.5$, $n_{\text{dividing}} = 22$, $n_{\text{not dividing}} = 2$, $p = 0.249$, 4µg/ml tetracycline: Mann-Whitney $U = 97.5$, $n_{\text{dividing}} = 9$, $n_{\text{not dividing}} = 20$, $p = 0.74$). However, the sample size for the control treatment ($n_{\text{not dividing}} = 2$) is too low to draw firm conclusions. Thick and thin lines indicates the non parametric population estimate and the upper and lower bounds of the 95% confidence interval for groups with 5 or more samples. Line and symbols above the data indicate results of a two sided Mann-Whitney $U$ test comparing the groups. n.s.: not significant ($p \geq 0.05$).
References

Appendix C

The cost of antibiotic resistance depends on evolutionary history in *Escherichia coli*
C.1 Relative Fitness of all genotypes
Table C.1  **Relative fitness of all genotypes.** Fitness was measured by competition assays against the wild type. Rifampicin-resistance (Rif<sup>r</sup>) mutations are given by their amino acid changes on rpoB; additional resistance elements (Str<sup>r</sup> or Sul<sup>r</sup>+Str<sup>r</sup>) are given either as amino acid changes on rpsL (K43N or K88R) or RSF1010 for the plasmid. Independently generated isolates of the wild type (evolved in LB or with additional resistance elements) are denoted a-c in Genotype name. Scores are mean ± s.e. from three independent replicates, or nine replicates across three blocks for genotypes with no additional resistance elements, and 27 replicates across three blocks of assays for the wild type. For WT<sub>c</sub>+K43N data are from one competition assay.

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<td>Evolved in LB</td>
<td>Additional\textsuperscript{a} element</td>
<td>Fitness</td>
<td>s.e.</td>
</tr>
<tr>
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<td>0.01</td>
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<td>none</td>
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<tr>
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<td>1.08</td>
<td>0.01</td>
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</table>
### Table C.1  Relative fitness of all genotypes. continued

<table>
<thead>
<tr>
<th>Genotype name</th>
<th>Rif(^{r}) mutation</th>
<th>Evolved in LB</th>
<th>Additional(^{l}) element</th>
<th>Fitness</th>
<th>s.e.</th>
</tr>
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<tr>
<td>WTc_evo</td>
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<td>yes</td>
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<td>1.13</td>
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<td>none</td>
<td>yes</td>
<td>RSF1010</td>
<td>1.18</td>
<td>0.01</td>
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</tbody>
</table>
C.2 Epistasis between RifR mutations, StrR mutations and plasmid RSF1010

Table C.2 Epistasis between RifR mutations, StrR mutations and plasmid RSF1010. We used a multiplicative model to estimate epistasis, $\epsilon$, for each pair of mutations or each mutation+plasmid combination in genotypes that had not adapted to LB; $\epsilon = W_{AB}W_{ab} - W_{Ab}W_{aB}$, where $W_{ij}$ is the fitness of genotypes with alleles $i$ and $j$ and capitals denote wild-type alleles (Trindade et al., 2009). To test whether epistasis values were significantly different from zero we used the error propagation method described in Trindade et al. (2009); asterisks denote significant deviations from multiplicative fitness effects. To estimate the fitness effect of a given resistance element in the wild type, we pooled the data for the three independently constructed strains, because they showed no significant variation in fitness and we therefore assume them to be genetically identical. To test whether these results were robust to a different model of epistasis, we estimated $\epsilon$ for the same data using an additive model, where $\epsilon = \alpha_{ab} - (\alpha_{Ab} + \alpha_{aB})$, where $\alpha$ is the fitness effect of having alleles $i$ and $j$ (Trindade et al., 2009; Phillips, 2008). Under an additive model we obtained the same qualitative results as with the multiplicative model, and scores from different models were closely correlated ($r^2 = 0.99$).

<table>
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<tr>
<th>Double mutant</th>
<th>Observed fitness ($w$)</th>
<th>s.d. $w$</th>
<th>Epistasis ($\epsilon$)</th>
<th>s.d. $\epsilon$</th>
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</thead>
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<tr>
<td>D516G+K88R</td>
<td>0.897</td>
<td>0.055</td>
<td>-0.069</td>
<td>0.082</td>
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<tr>
<td>I572S+K43N</td>
<td>0.936</td>
<td>0.019</td>
<td>0.057*</td>
<td>0.048</td>
</tr>
<tr>
<td>S512F+K43N</td>
<td>0.806</td>
<td>0.019</td>
<td>-0.007</td>
<td>0.053</td>
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<tr>
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<td>0.935</td>
<td>0.028</td>
<td>-0.050</td>
<td>0.064</td>
</tr>
<tr>
<td>D516G+plasmid</td>
<td>0.909</td>
<td>0.037</td>
<td>-0.126*</td>
<td>0.115</td>
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<tr>
<td>S512F+plasmid</td>
<td>1.000</td>
<td>0.024</td>
<td>0.007</td>
<td>0.063</td>
</tr>
<tr>
<td>I572S+plasmid</td>
<td>1.056</td>
<td>0.004</td>
<td>-0.067*</td>
<td>0.065</td>
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</tbody>
</table>
C.3 Chaperone levels in different genotypes

**Fig. C.1** Chaperone levels in different genotypes. Protein levels were determined by western blots for all genotypes relative to the wild type (1.0). Data are shown for two molecular chaperones (a) DnaK and (b) GroEL for each genotype (x-axis) before (white bars) and after (hatched bars) evolution in LB. Bars show standard errors. Neither chaperone was overexpressed after evolution (main text).
References


Appendix D

Curriculum vitae
Curriculum vitae

Daniel Christoph Angst, born December 13th, 1984
citizen of Rheinau, ZH, Switzerland.
Grundstrasse 10, 8048 Zürich, Switzerland. dcangst@gmail.com

Education

2011 - 2016  **PhD, ETH Zürich**
with Prof. Dr. Sebastian Bonhoeffer (Theoretical Biology) and Prof. Dr. Martin Ackermann (Molecular Microbial Ecology)

2009 - 2011  **MSc Environmental Sciences, ETH Zürich**
Major in Ecology and Evolution

2005 - 2008  **BSc Environmental Sciences, ETH Zürich**

Work Experience

2010 - 2011  **Internship at Actelion Pharmaceuticals Ltd.**
Group Anti-Infectives, Task area: in vitro characterization of the antibacterial properties of novel pharmaceutical compounds.

2008 - 2010  **Asisstant to the Directorate, Wasser-Agenda 21**
(Association for the sustainable development of the swiss hydropower sector, residing at Eawag, the Swiss Federal Institute of Aquatic Science and Technology)

2007 - 2009  **Student Assistant, ETH Zürich**
Chair of Sustainability and Technology (SUSTEC, Prof. Dr. Volker Hoffman) Department of Management, Technology and Economics (D-MTEC)

Extracurricular activity

2013 - 2013  **Lab manager at FabLab Zürich. Instructor for digital manufacturing technologies such as 3D printing, laser cutting and CNC machining.**
APPENDIX D. CURRICULUM VITAE

2010  Student member of the Search Committee for the Chair of Evolutionary Biology at ETH Zürich.

2008 - 2010  Student representative in the teaching commission of the Department for Environmental Sciences at ETH Zürich.

Publications

Shilaih, M.*, Angst, D. C.*, Marzel, A., Bonhoeffer, S., Günthard, H., Kouyos, R. Antibacterial Effects of Antiretrovirals, Implications for Microbiome Studies in HIV. *these authors contributed equally to the work. (under review)


Conference Presentations

2015  Angst, D. C., Ackermann S., Bonhoeffer S. Measuring cost/benefit trade-offs of tetA expression. Bacterial Networks (Bacnet), San Feliu de Guixols, Spain. *(Poster)*

2014  Angst, D. C., Ackermann S., Bonhoeffer S. Cost/Benefit Tradeoffs of tetA Expression. Swiss Meeting for Infectious Disease Dynamics (SMIDDY) 2014, Bern, Switzerland. *(Talk)*


2013  Angst, D. C., Ackermann S., Bonhoeffer S. Disentangling the costs and benefits of resistance. New Approaches and Concepts in Microbiology, EMBL Heidelberg, Germany *(Poster)*
2012  Angst, D. C., Ackermann S., Bonhoeffer S. Gene Regulation and the costs of antibiotic resistance. ISME Conference 2012, Copenhagen, Denmark (*Poster*)