Effect of multiple-antibiotic treatments on the evolution of antibiotic resistance in *Pseudomonas aeruginosa*

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A thesis submitted for the degree of
Doctor of Philosophy

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Abstract

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To combat the ever-growing clinical burden imposed by antibiotic-resistant pathogens, multiple-antibiotic treatments are increasingly being considered as promising treatment options. The impact of multiple-antibiotic treatments on the evolution of resistance is not well understood however, and debate is ongoing about the effectiveness of various multiple-antibiotic treatments. In this thesis, I investigate how aspects of multiple-antibiotic treatments impact the rate of evolution of antibiotic resistance in the opportunistic human pathogen *Pseudomonas aeruginosa.* In particular, I look at the impact of interactions between antibiotics in combination on the evolution of resistance, and how creating heterogeneity in the antibiotic environment by rotating the antibiotics used may change the rate of evolution of resistance.

I characterise the interactions present in 120 combinations of antibiotics and find that the type of interaction can be predicted by the mechanism of action of the antibiotics involved. I investigate the effect of a subset of these combinations on the evolution of antibiotic resistance. My results refute the influential but poorly-evidenced hypothesis that synergistic combinations accelerate the evolution of resistance, even when synergistic combinations have the same inhibitory effect on sensitive bacteria as additive or antagonistic antibiotic combinations. I focus on a combination of the antibiotics ceftriaxone and sulfamethoxazole and test whether it is more effective in preventing the evolution of resistance than predicted by the inhibitory effect of the combination on sensitive bacteria. I do not find the combination to be more effective than predicted.

Finally, I create heterogeneous antibiotic environments by rotating the antibiotic present at different rates. For the first time in a laboratory setting, I test how varying the rate of fluctuation in the antibiotics present in a heterogeneous antibiotic environment impacts the rate of evolution of resistance. Unexpectedly, I find the rate of evolution of resistance increases with increasing levels of antibiotic heterogeneity.

Declaration of Authorship

I intend to publish the following papers based on the work presented in this thesis:

Whiteley, R. and MacLean, R.C., Interactions between antibiotics do not influence the rate of evolution of resistance in *Pseudomonas aeruginosa* (manuscript in prep., based on Chapter 3 of this thesis)

Whiteley, R., Betts, A., Furio, V., Gifford, D.R., Heilbron, K., Qi, Q., San Millan, A., Vogwill, T. and MacLean, R.C., Higher levels of antibiotic heterogeneity accelerate the evolution of antibiotic resistance, in a *Pseudomonas aeruginosa* experimental system (manuscript in prep., based on Chapter 5 of this thesis but to incorporate additional experimental data produced in collaboration with the other authors)

I hereby certify that the work in this thesis is wholly my own, with the following exceptions:

Chapter 2: I received useful comments from Craig MacLean which were incorporated into this chapter.

Chapter 3: The text was written by me with input from Craig MacLean.

Chapter 4: I received useful comments from Craig MacLean which were incorporated into this chapter.

Chapter 5: The text was written by me with input from Craig MacLean.

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Chapter 1

1 Introduction

1.1 The challenge of antibiotic resistance

When antibiotics were introduced into clinical use, many believed bacterial disease would be consigned to history [1]. Infections which might previously have been fatal could now be easily treated, and antibiotics allowed the development of new forms of surgery [2]. Unfortunately, the first signs of trouble came not long after the first introduction of antibiotics, with sulphonamide resistance presenting a clinical problem shortly after the introduction of sulphonamide antibiotics in the 1930s [3]. Since then, each time a novel antibiotic has been deployed to clinical use, bacteria resistant to the antibiotic have been isolated, sometimes within a few years or less [4] (see Fig. 1.1). Antibiotic-resistant infections are associated with greater mortality, longer hospital stays and more costly care compared to antibiotic-sensitive infections [5, 6]. Bacterial resistance to antibiotics is now acknowledged as a major threat to public health globally [1, 7-9], with one estimate by the Infectious Disease Society of America placing the proportion of hospital-acquired infections in the US which are antibiotic-resistant at 70% [4].

Of particular concern are bacteria which have evolved resistance to more than one antibiotic; they are multiple-antibiotic resistant. Such pathogens increase the chance of patients being given an ineffective antibiotic treatment and are associated with adverse

clinical outcomes [10]. Pathogens have been isolated which are pan-antibiotic resistant; they are resistant to all possible antibiotic treatments, raising concerns of the onset of a 'post-antibiotic era' [8, 11, 12].

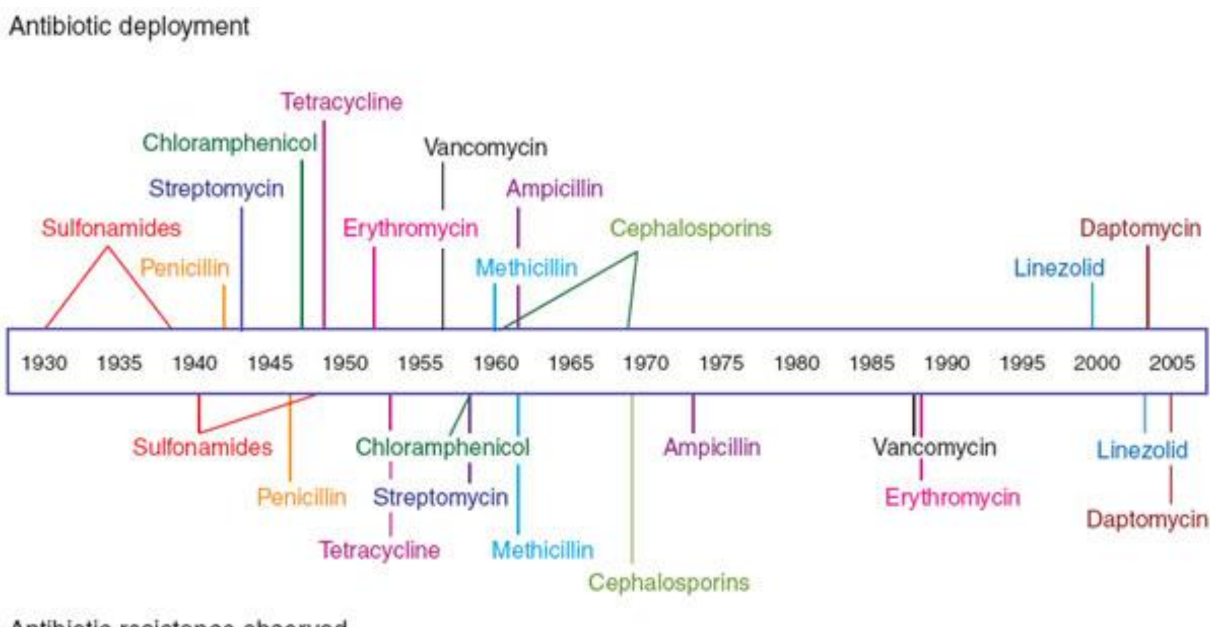


Figure 1.1. Year of antibiotic deployment and year antibiotic resistance was first observed, for a number of clinically important antibiotics. Reprinted by permission from Macmillan Publishers Ltd: Nature Chemical Biology, Clatworthy, A.E., Pierson, E. and Hung, D.T., *Targeting virulence: a new paradigm for antimicrobial therapy.* Nat Chem Biol, 2007. **3**(9): p. 541-548 [4]. Copyright 2007.

Faced with the development of antibiotic resistance, pharmaceutical companies at first searched for new antibiotics to which bacteria did not have resistance, and produced semi-synthetic derivatives of existing antibiotics which could circumvent existing resistance mechanisms [1]. Resistance to these novel antibiotics soon emerged, however. The pharmaceutical industry has now largely withdrawn from the field of antibiotic discovery for economic reasons, leading to a drying up of the 'drug-supply pipeline'. Concerned organisations are leading efforts to revive antibiotic discovery (see

for example [13, 14]), but in addition alternatives to antibiotic treatment need to be considered, as well as how to best preserve the usefulness of the antibiotics we have. Alternatives include the development of vaccines against bacterial diseases, combining antibiotics with inhibitors of antibiotic-resistance mechanisms (as in the case of co-amoxiclav, a treatment which combines amoxicillin, a β-lactam antibiotic, with clavulanic acid, a β-lactamase inhibitor), and therapy with bacteriophages [1].

Much clinical focus has been given to preserving the usefulness of existing antibiotics. This includes preventing inappropriate antibiotic use (for example prescribing antibiotics for viral infections, which antibiotics cannot treat), and minimising the spread of resistant pathogens through infection control measures [7]. In hospitals, the use of multiple-antibiotic treatments is being considered as a promising option to deal with resistant pathogens, or to prevent the initial evolution of resistance [9, 15-17]. Such multiple-antibiotic treatments include using antibiotics in combination and creating heterogeneity in the antibiotics present in the environment. (The rationale behind each of these strategies is explained in more detail later in this thesis.) Finally, roughly half of antibiotics produced worldwide are used in agriculture and aquaculture rather than in the clinic [1]; this represents an unnecessary opportunity for bacterial strains, which may later infect humans or transfer resistance elements to human pathogens, to acquire antibiotic resistance [7, 18, 19]. Efforts are being made to reduce the use of antibiotics in agriculture [20].

1.2 Mechanisms of antibiotic action and resistance

In order to inhibit bacterial pathogens without harming the human host, antibiotics make use of biochemical differences between prokaryotic and eukaryotic cells [21]. I set out the mechanisms of action of some commonly used antibiotic families below.

1.2.1 Mechanisms of action of antibiotics

B-lactam antibiotics

B-lactam antibiotics target bacterial cell-wall synthesis. In the bacterial cell wall, the peptidoglycan layer confers strength; the peptide strands are cross-linked by the action of transpeptidases. B-lactam antibiotics competitively bind to the active site of the transpeptidases (the penicillin-binding protein) as pseudosubstrates, and then acylate the active site, a process which reverses only very slowly. By preventing transpeptidases from crosslinking peptide chains, β-lactams make a cell mechanically weak and vulnerable to osmotic lysis [21-23].

Aminoglycosides

Aminoglycoside antibiotics inhibit bacterial protein synthesis. They bind to the 30S ribosomal subunit, and thereby disrupt the proof reading process which ensures accurate translation, resulting in misreading and premature termination. Thus malformed proteins are produced [24].

Quinolones

Fluoroquinolone antibiotics are synthetic antibiotics which inhibit DNA replication. They target the enzyme DNA gyrase, which uncoils double stranded bacterial DNA. By forming a complex with DNA gyrase and the double stranded DNA tethered to it, quinolones prevent the proper action of DNA gyrase [21, 23].

Polymyxins

Polymyxin antibiotics act by disintegrating cell membranes. They are natural antimicrobials found in eukaryotic cells, and are also known as cationic antimicrobial peptides. Polymyxins bind to lipid A in lipopolysaccharide, a structural component of the bacterial outer membrane. The binding of polymyxins to lipid A results in the destabilisation and disruption of the inner and outer membranes [25].

Sulphonamides

Sulphonamide antibiotics inhibit folate synthesis. Sulphonamides inhibit the action of dihydropteroate synthase, as they are pseudosubstrates of one of the enzyme's substrates, ρ- aminobenzoic acid [26]. By disrupting this step in folate synthesis, they ultimately inhibit protein synthesis and other metabolic processes [27, 28].

Rifamycins

Rifamycins bind to RNA polymerase and block transcription, thus inhibiting protein synthesis [29].

Bactericidal and bacteriostatic antibiotics

Antibiotics may be divided into those that act through a bactericidal mechanism and those that act through a bacteriostatic mechanism. Bactericidal antibiotics kill bacteria, whereas bacteriostatic antibiotics inhibit growth of bacteria but do not kill them so bacterial growth may resume when the antibiotic is removed. Β-lactam, aminoglycoside, quinolone and polymyxin antibiotics are usually thought of as bactericidal antibiotics, and sulphonamide and rifamycin antibiotics as bacteriostatic antibiotics. However, whether an antibiotic is bactericidal or bacteriostatic may be concentration dependent [30]; for example low concentrations of quinolone antibiotics have a bacteriostatic action [23].

1.2.2 Mechanisms of antibiotic resistance

There are a number of mechanisms by which bacteria may overcome the inhibitory effect of antibiotics (see Table 1.1 for a description of some resistance mechanisms against each antibiotic family described above). These mechanisms may be divided into three broad classes: inactivating the antibiotic, altering antibiotic target sites so they are no longer susceptible to the antibiotic, and preventing the antibiotic from reaching a high intracellular concentration.

Bacteria may produce enzymes which inactivate the antibiotic. For example, β-lactam resistant bacteria may produce β-lactamase, which deactivates the β-lactam ring in penicillin and cephalosporin β-lactam antibiotics. Thus the antibiotic is rendered ineffective [21]. Another resistance strategy is alteration of the structure of the sites that

antibiotics bind to, to reduce the affinity of antibiotics for these sites. The bacterial RNA polymerase β-subunit, which is targeted by rifamycin antibiotics, is encoded by the *rpoB* gene. Amino acid substitutions at a limited number of highly conserved sites in *rpoB* confer high level resistance to rifamycins. These substitutions alter the structure of the β-subunit, reducing its affinity for rifamycins [31]. Bacteria may also adopt a strategy of actively removing antibiotic molecules from the cell, in order to prevent intra-cellular antibiotic concentrations reaching a harmful level. Efflux pumps are proteins that actively transport toxic substances from within cells to the external environment. Some efflux pumps transport a broad range of substances. For example, when expressed at a high level, the MexAB pump in *Pseudomonas aeruginosa* reduces susceptibility to β-lactams, fluoroquinolones, the antibiotics chloramphenicol and trimethoprim, and the household biocide triclosan [32]. Therefore, the *mexAB* pump system confers multiple-antibiotic resistance.

Antibiotic family	**Mechanism of action**	**Mechanisms of resistance**
B-lactams	Bind to penicillin-binding proteins and prevent cross-linking in cell wall	B-lactamases degrade the antibiotic Modification of penicillin-binding protein site so antibiotic does not bind Efflux pumps
Aminoglycosides	Inhibit protein synthesis	Enzymatic modification of the antibiotic Efflux pumps
Quinolones	Inhibit DNA replication by binding to DNA gyrase	Alteration of structure of DNA gyrase Efflux pumps

Polymyxins	Disintegrate cell membrane	Modification of the outer membrane structure Efflux pumps
Sulphonamides	Inhibit folate synthesis	Altered enzyme structure which binds to ρ-aminobenzoic acid but not sulphonamides
Rifamycins	Inhibit transcription by binding to RNA polymerase	Alteration of structure of β-subunit of RNA polymerase, through mutations in *rpoB*

Table 1.1. Mechanism of action and some mechanisms of resistance for various antibiotic families.

1.2.3 *De novo* and horizontally acquired resistance mechanisms

When resistance mechanisms are found in a bacterial strain, the mutations conferring resistance may have arisen in that strain (*de novo* resistance). Alternatively, the resistance-conferring mutations may have arisen in another strain or another bacterial species, and have been transferred to the strain being studied by horizontal gene transfer. For example, plasmids may transfer resistance genes through bacterial populations. Plasmids often carry several genes, with each gene conferring resistance to a different antibiotic. Treatment of plasmid-carrying bacteria with any one of the antibiotics the plasmid confers resistance to can therefore promote the spread of the plasmid, and the spread of resistance to all the antibiotics the plasmid carries resistance to [33].

The relative importance of *de novo* resistance and horizontally acquired resistance varies amongst species [33, 34]. It is thought that during the course of antibiotic treatment of an individual patient, resistance arises mainly through mutation [35]. Ultimately, all horizontally acquired resistance mutations have arisen through *de novo* mutation at some point. In this thesis, I focus on *de novo* chromosomal resistance.

1.3 Factors affecting the rate of evolution of *de novo* antibiotic resistance

The process by which a bacterial population, challenged with an antibiotic to which it is susceptible, evolves antibiotic resistance may be split into a stochastic and a deterministic stage [33]. In the stochastic stage, a resistance mutation must appear in the population and increase in frequency until it is not in danger of loss through genetic drift. The change in frequency is determined by genetic drift and by the selection coefficient. In the deterministic stage, selection further increases the frequency of the mutation until it is fixed in the population or reaches an equilibrium proportion, with the rate of increase dependent largely on the selection coefficient (see Fig. 1.2). A number of factors may determine the rate at which resistance increases in the population during each stage.

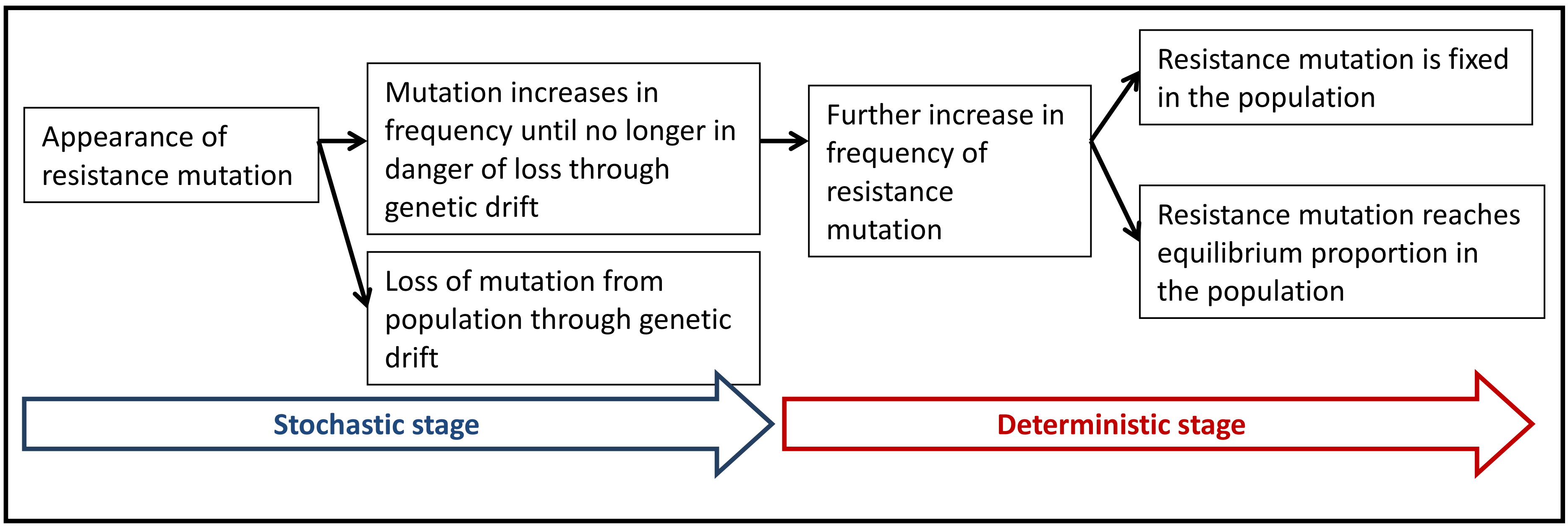


Figure 1.2. Diagrammatic representation of the process through which a single copy of a resistance mutation may reach high frequency in a bacterial population undergoing antibiotic treatment.

1.3.1 Evolution of resistance under continuous exposure to one antibiotic

First, I consider the factors that determine the rate of evolution of antibiotic resistance in the simple case of continuous exposure to one antibiotic.

Rate of resistance-conferring mutation

When a bacterial population is treated with an antibiotic, selection will act on the variation already present in the population [33]. Resistant mutants may already be present in the population at a low frequency. In some cases, more than one mutation may need to arise sequentially in the same clone in order to confer a high level of resistance [9], and populations will need to 'find' further mutations after antibiotic treatment has begun. The supply rate of resistance mutations is the product of the intrinsic mutation rate and the population size. A higher supply rate of resistance mutations will accelerate the evolution of antibiotic resistance.

The intrinsic rate of resistance-conferring mutation may be different for different antibiotics. This is because there may be more target sites at which mutation confers resistance for some antibiotics than others [9, 36, 37]. In addition, mutation rate may differ between bacterial strains and species. Mutators are bacteria with an elevated mutation rate, often due to defects in the methyl-directed mismatch repair system [38]. Mutators are often isolated from patients who have received extensive antibiotic treatment, for example cystic fibrosis patients [39, 40]; elevated mutation rate may be beneficial in this environment as it increases the rate of evolution of antibiotic resistance [41-43]. The environment in which bacteria find themselves may also alter

the mutation rate; for example, antibiotics which cause DNA damage may trigger the SOS response and lead to an increased mutation rate [44].

Population size

Larger populations may evolve antibiotic resistance more rapidly. The greater the population size, the higher the mutation supply rate (i.e. the product of the intrinsic mutation rate and the population size). Therefore, larger populations are likely to 'find' resistance mutations more quickly. The larger the absolute population size, the less likely a mutation is to be lost from the population by genetic drift. In a small absolute population size, a mutation may arise many times before it, by chance, escapes extinction by genetic drift and is able to enter the deterministic stage [33]. The higher the frequency of a mutation (i.e. the higher the proportion of the population that is made up by mutants), the less likely the mutation is to be lost by genetic drift.

In both the stochastic and the deterministic stage, the rate of increase in the frequency of resistance mutations is dependent on the selection coefficient [33]. The inhibitory effect of antibiotics can vary at different population sizes; a given concentration of antibiotics may be less effective at inhibiting cells in a larger bacterial population [45-47].

Antibiotic concentration

Higher antibiotic concentrations may accelerate or decelerate the evolution of antibiotic resistance. It may be possible to achieve a concentration of an antibiotic so high that it overcomes the resistance mechanisms even of highly resistant bacteria; such concentrations are above the 'mutant prevention concentration' [48]. In this case, as all bacteria are inhibited, the high concentration clearly prevents any evolution of resistance. Concentrations above the mutant prevention concentration are ideal clinically, but are not always possible because of toxicity to the patient [49, 50]. In addition, antibiotics struggle to reach certain areas of the body, creating lower concentration 'refuges' where resistance may evolve [51], and antibiotic concentration will vary over time dependent on the dosing regime [52]. The other extreme is when no antibiotic is used; there will be no selection pressure for resistance and so we would not expect antibiotic resistance to evolve.

Antibiotic concentrations achieved clinically may often fall within the 'mutant selection window'. This is the range of antibiotic concentrations at which resistant mutants can survive and grow, but sensitive wild type bacteria cannot (concentrations above the minimum inhibitory concentration or MIC for the wild type sensitive strain, but below the mutant prevention concentration) [48]. At these concentrations, the selection pressure for resistance is very strong (in fact, the selection coefficient is 1) as only resistant mutants will replicate.

In other cases, the antibiotic concentration may be below the MIC for the wild type sensitive strain, but the antibiotic impairs the growth of the sensitive strain even though it does not completely inhibit it. In this scenario, antibiotic resistant mutants may still have a selective advantage over the sensitive strain, if they are able to grow and divide more quickly in the presence of the antibiotic [53]. The greater the selective advantage of the resistant mutants over the sensitive wild type strain, the quicker they increase in frequency. Increasing the sub wild-type MIC concentration of an antibiotic is likely to increase the selective advantage of the resistant mutants over the sensitive strain and accelerate the evolution of antibiotic resistance. However, higher antibiotic concentrations will also reduce the population size of the sensitive strain, which may reduce the rate of supply of resistance conferring mutations, as discussed above. The relative importance of a higher antibiotic concentration in accelerating evolution by increasing the selective advantage of resistance, and suppressing evolution by reducing the supply of resistance mutations, is likely to vary between different scenarios (see Chapter 6 for a discussion of how this issue affected my experimental system).

In this thesis I focus on antibiotic concentrations which are below the MIC of the sensitive wild type strain, so that I am able to quantify interactions between antibiotics in Chapters 2 and 3. The implications of focusing on sub-wild type MIC antibiotic concentrations are discussed in Chapter 6.

Cost of resistance

Antibiotic resistance will usually, though not always, carry a fitness cost. This means that the resistant mutant will grow less well than the sensitive wild type strain in the absence of antibiotics. Costs arise because antibiotics target critical and highly conserved cellular functions [54], and so alterations through resistance conferring mutations tend to reduce the efficiency of cell functioning [55, 56]. Where the antibiotic concentration used is lethal to the sensitive wild type strain, antibiotic resistant mutants will have a selective advantage regardless of how costly resistance is, and high-cost mutations may increase to high frequency in the population [57]. Where the antibiotic concentration only partially inhibits sensitive bacteria, resistant mutants with very costly mutations may not replicate more quickly than sensitive bacteria and may not have a selective advantage. The greater the cost of resistance, the less the selective advantage of resistance and so the more slowly resistance will evolve.

Antibiotic resistant mutants often acquire compensatory mutations [55, 58-60]. These are secondary mutations which reduce the cost of resistance. Once a resistant mutant has acquired compensatory mutations, resistance will be less costly or even free of cost [61], increasing the selective advantage of resistant mutants and the rate of evolution of resistance. Positive epistasis between resistance mutations is common [62], so that the cost of acquiring further resistance mutations to increase the level of resistance is reduced in a mutant already containing one resistance mutation. Epistasis may therefore increase the rate of evolution of high level resistance.

Clonal interference

As bacteria reproduce clonally, if two resistance mutations arise in two different clones, the mutations cannot be brought together by recombination. Instead, both resistant clones may enjoy a selective advantage over the sensitive wild type strain. The two clones will compete with each other, delaying the fixation of the highest fitness clone and thus reducing the rate of evolution of antibiotic resistance [33]. Amongst clinical *Mycobacterium tuberculosis* isolates, no or low cost rifampicin resistant mutant strains are the most frequent resistant strains [63], suggesting that competition between resistant strains is common in clinical scenarios.

Environmental factors

A number of environmental factors other than the presence of an antibiotic can affect the rate of evolution of antibiotic resistance. For example, in the laboratory the MIC can vary depending on the richness of the culture medium used, the concentration of magnesium and calcium ions it contains, and the PH of the environment [64]. Temperature may also affect bacterial susceptibility to antibiotics [65].

1.3.2 Additional factors affecting the rate of evolution of resistance when multiple-antibiotic treatments are used

In this thesis, I focus on the effects of multiple-antibiotic treatments on the rate of evolution of antibiotic resistance. In addition to the factors affecting the rate of evolution of resistance to single continuously applied antibiotics, there are a number of further considerations when using multiple-antibiotic treatments, as I discuss below.

Interactions between antibiotics

A combination treatment is where bacteria encounter two or more antibiotics in the environment together (so, in a clinical scenario, a patient is given two or more antibiotics simultaneously). An important consideration for clinicians using antibiotic combinations is that the antibiotics may interact and alter each other's effect on bacterial growth and replication. Combinations may interact synergistically, where the inhibitory effect of two antibiotics in combination is greater than would be expected if the two antibiotics acted independently of each other, or antagonistically, where the inhibitory effect of two antibiotics in combination is less than would be expected if the two antibiotics acted independently of each other (see Figs. 1.3 and 1.4). In Chapter 2 of this thesis, I characterise all pairwise interactions between a set of 16 antibiotics, and identify trends in the interactions shown.

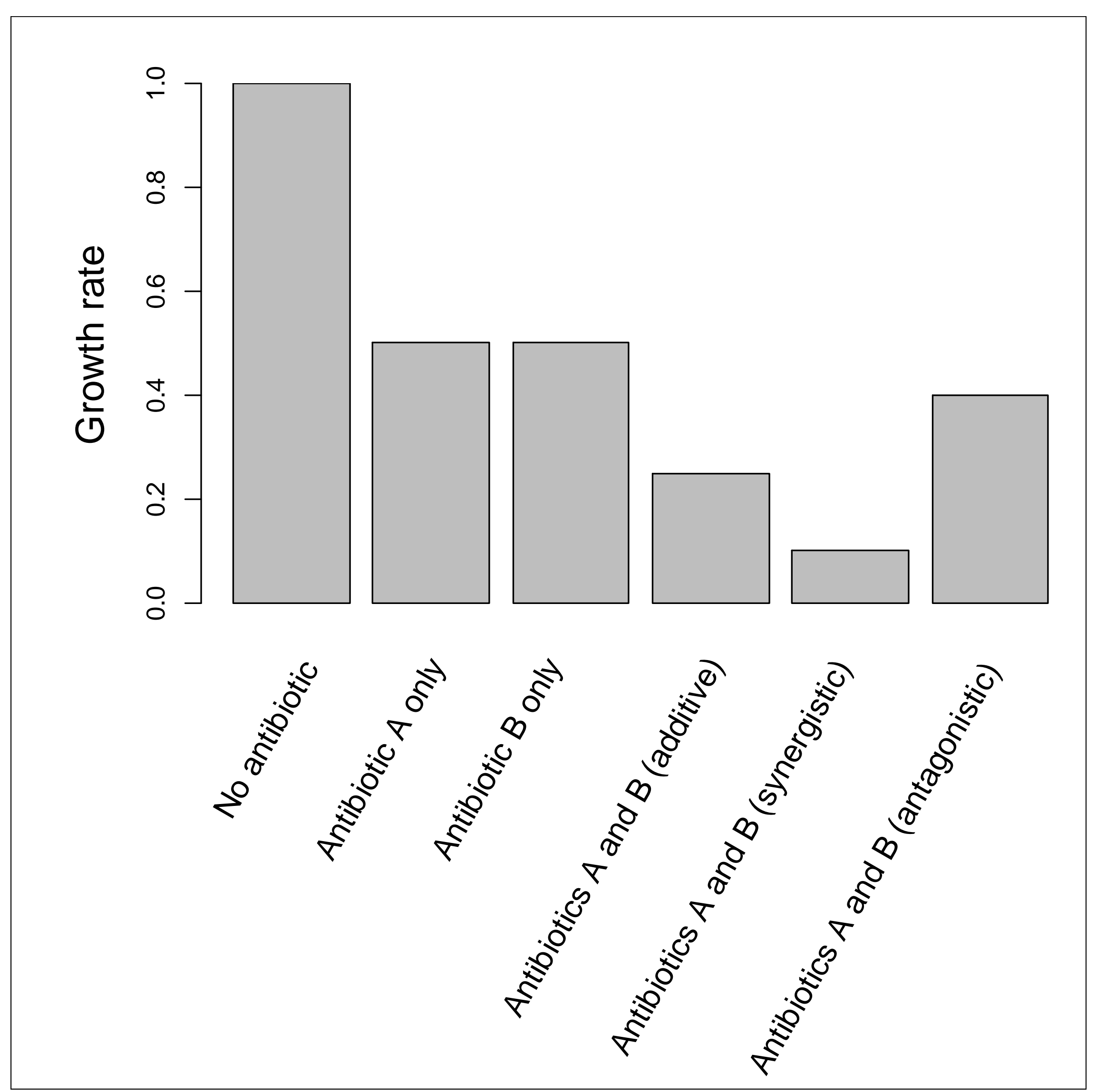


Figure 1.3. Illustrative growth rates of bacteria in various environments containing single antibiotics or combinations of antibiotics at sub- MIC concentrations (type of interaction between antibiotics in a combination given in brackets). The additive model is a null model which assumes no interaction between antibiotics. Note that the growth rates for the synergistic and antagonistic combinations are illustrative; a combination producing a growth rate higher than 0 but less than the growth rate of the additive combination would be characterised as synergistic. A combination producing a growth rate higher than than of the additive combination would be characterised as antagonistic (with a combination producing a growth rate higher than either antibiotic on its own characterised as hyper-antagonistic).

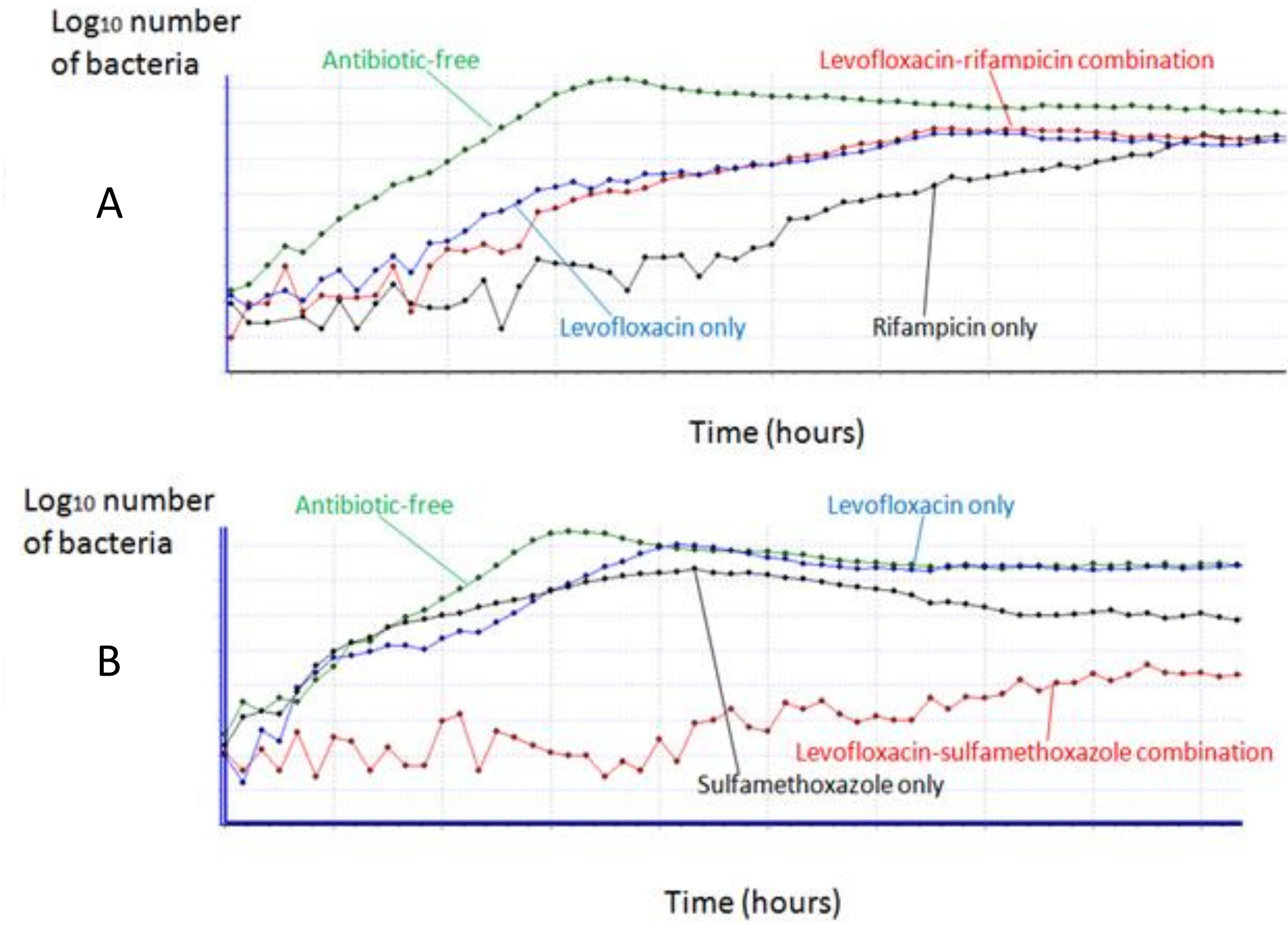


Figure 1.4. Illustrative examples of interactions between antibiotics in combination. Panel *(A)* shows an example of an antagonistic combination of antibiotics (levofloxacin and rifampicin). Levofloxacin appears to provide a protective effect against rifampicin, as bacteria grow faster with levofloxacin and rifampicin in combination than with only rifampicin. Panel *(B)* shows an example of a synergistic combination of antibiotics (levofloxacin and sulfamethoxazole). The inhibitory effect of levofloxacin and sulfamethoxazole in combination is greater than we would predict by multiplying together the inhibitory effect of each antibiotic on its own. Figure prepared with data from work undertaken in Chapter 2.

In general, clinicians favour synergistic combinations of antibiotics as they are more effective at inhibiting bacteria for a given concentration of antibiotics. Thus they increase the chance of infection being cleared [66], while allowing lower concentrations to be used, minimising side effects for patients [67] and costs. Antagonistic

combinations are generally avoided clinically. However, it has recently been suggested that synergistic combinations may actually accelerate the evolution of antibiotic resistance, whereas antagonistic combinations may delay or prevent the evolution of resistance [68].

Higher antibiotic concentrations accelerate the evolution of antibiotic resistance through their greater inhibitory effect on the sensitive strain, thus increasing the selective advantage of the resistant mutants over the sensitive strain. In a similar way, synergistic combinations are more inhibitory to the sensitive strain than antagonistic combinations, and so increase the selective advantage of resistant mutants. In addition to accelerating the rate of evolution of resistance through differential effects on the sensitive wild type strain, Hegreness *et al.* have proposed that the type of interaction may also influence the rate of evolution of resistance through differential effects on mutants resistant to one antibiotic in a combination [68]. Singly-resistant mutants may gain a larger selective advantage over the sensitive strain when exposed to a synergistic combination than an antagonistic combination, thereby further accelerating the evolution of resistance to synergistic combinations of antibiotics. I explain this hypothesis in detail and explore its validity in Chapter 3.

Note that there are two alternative methods for assessing whether antibiotics in combination are interacting in a synergistic or antagonistic manner. Two alternative models can be used to calculate the null-hypothesis growth rate that would be expected if the antibiotics in a combination do not interact. Deviations from this null expectation can then be interpreted as antagonistic or synergistic interactions between the

antibiotics in combination. In the Loewe additivity model, it is assumed that each antibiotic in a combination has a similar mode of action [103]. The effect of non-interacting antibiotics in combination will be the sum of the inhibitory effect of each antibiotic individually. This null model therefore assumes that dose-response curves are linear. In contrast, the Bliss independence model, a multiplicative model, assumes the action of each antibiotic in a combination is independent [103, 115]. The effect of non-interacting antibiotics in combination will be the product of the inhibitory effect of each antibiotic individually. For my experiments, I used the Bliss independence model as I was using antibiotics from different antibiotic families and therefore with very different modes of action. Note that the Bliss independence model cannot be used to predict the effect of increasing the dose of a single antibiotic, as the model assumes that each component of a mixture has a different mode of action. Due to the way the null-hypothesis growth rate is calculated by each model, the Loewe additivity model expects lower bacterial growth rates than the Bliss independence model. Therefore the Loewe additivity model tends to find more combinations of antibiotics to interact antagonistically than the Bliss independence model.

Antibiotic heterogeneity

An alternative multiple-antibiotic strategy to combination therapy is to create antibiotic heterogeneity. This is where spatial or temporal heterogeneity in the antibiotics bacteria encounter is created. Different patients in a hospital may be assigned different antibiotics (a ‘mixing’ strategy), so that pathogens colonising a new host may find the antibiotic treatment is different than the one they had previously encountered. Alternatively, all patients in a ward may be given the same antibiotic, and the antibiotic

used is rotated periodically, eventually returning to the first antibiotic (antibiotic ‘cycling’) so that pathogens encounter different antibiotics over time. The aim of creating heterogeneity is to reduce the selection pressure for resistance to any one antibiotic and thus reduce the rate of evolution of resistance to any one antibiotic. It is also hoped that antibiotic resistant strains may reduce in frequency when they compete with antibiotic sensitive strains in the absence of a particular antibiotic, reducing the long term accumulation of resistance amongst bacterial populations.

There has been considerable discussion about the relative effectiveness of different antibiotic heterogeneity strategies in reducing the rate of evolution of antibiotic resistance [15, 69, 70], and about how antibiotic heterogeneity compares to combination therapy [71, 72]. In general, mathematical models have suggested that the more heterogeneous an environment, the slower the rate of evolution of antibiotic resistance [15, 73], whereas clinical studies have produced conflicting results [15]. Few laboratory-based studies have addressed this issue. In Chapter 5 I conduct, for the first time, an experimental evolution study into how environments with different degrees of antibiotic heterogeneity affect the rate of evolution of antibiotic resistance.

Multiple-antibiotic resistance, epistasis and cross-resistance

When exposed to a multiple-antibiotic treatment, bacteria may evolve resistance to all of the antibiotics in the treatment, and become multiple-antibiotic resistant. This may

occur through the acquisition of a single resistance mechanism that confers resistance to more than one antibiotic, for example upregulation of an efflux pump [32]. Alternatively, a bacterial strain may acquire separate mutations conferring resistance to each antibiotic. In this case, epistatic interactions may occur between resistance mutations, increasing or decreasing the cost of acquiring a further resistance mutation in a genetic background already containing a resistance mutation [33, 62, 74]. It is thought that positive epistasis may be common between resistance mutations, so that the cost of acquiring a second mutation is reduced in a resistant genetic background, thus accelerating the rate of evolution of multiple-antibiotic resistance [55, 62].

In heterogeneous treatments, where bacteria are exposed to multiple antibiotics sequentially, cross-resistance may occur. This is where a mutation conferring resistance to one antibiotic alters bacterial resistance to a secondary antibiotic not yet encountered. Positive cross-resistance is where evolving resistance to one antibiotic also increases resistance to the secondary antibiotic. Positive cross-resistance is of clinical significance, and may be common (see for example [75-81]). Negative cross-resistance, where evolving resistance to one antibiotic increases sensitivity to another antibiotic, has been suggested as a property that could be exploited to prevent the stable development of resistance [82]. Positive cross-resistance may therefore accelerate the evolution of multiple-antibiotic resistance, and negative cross-resistance may delay it.

1.4 Importance of experimental approach

Research into the effect of multiple-antibiotic treatments on the evolution of antibiotic resistance has been carried out using clinical trials, mathematical modelling and

experimental evolution studies. In experimental evolution, populations of organisms are propagated under controlled laboratory conditions over a number of generations, and changes in the phenotype or genotype of organisms investigated. Experimental evolution offers a number of advantages for investigations into the evolution of bacterial populations (as outlined in [83]):

- Environmental conditions can be controlled and easily manipulated in the test tube. Clinical studies cannot control conditions as tightly, for example because the rate of clearance of antibiotics from the body can vary between patients [84].
- The genetic composition of founding populations can be controlled.
- Ancestral bacteria can be preserved in the freezer and revived later, to allow comparisons between ancestral and evolved bacteria.
- As large populations can grow in small spaces, a high level of replication is possible.
- There are well developed techniques for manipulating and analysing microorganisms in experimental evolution studies.
- By working with living populations, experimental evolution is able to capture aspects of a system that may not be obvious and may not be included in mathematical models; for example whether some antibiotics are more difficult for bacteria to evolve resistance to than others.

Experimental evolution has provided many important insights into the process of evolution [85]. There are, however, drawbacks to using this approach to study the evolution of antibiotic resistance. In particular, experimental evolution creates a simple

model system that does not capture the complexity of the environment experienced by bacteria in clinical situations. For example, *in vitro* systems may not capture the effect of the immune system on the evolution of pathogens, the co-evolution of bacteria and bacteriophages within a host or the variation in the concentration of antibiotic present in different parts of the body or at times in a dosing regime. The applicability of results obtained through experimental evolution to clinical situations therefore needs to be treated with caution. Experimental evolution allows hypotheses about the evolution of antibiotic resistance to be tested relatively rapidly and cheaply, and promising results can then be investigated using *in vivo* systems.

1.5 *Pseudomonas aeruginosa* as a study organism

In this thesis, I conduct experimental evolution studies using the bacterium *Pseudomonas aeruginosa*. *P. aeruginosa* is a gram-negative, rod shaped bacterium with one of the largest prokaryotic genomes (the PA01 genome contains 6.3 million base pairs) that gives it the nutritional versatility to exploit many different environments [86]. It is commonly found in moist environments, for example the soil, water and in plants and animals [86], and strains have even been isolated growing in disinfectants [19] and fuel oil [1]. Importantly, *P. aeruginosa* is also an opportunistic human pathogen, commonly infecting patients with compromised natural defences, for example cystic fibrosis patients, burn wound patients and cancer patients receiving chemotherapy [87]. Infection with *P. aeruginosa* is associated with a poor clinical outcome relative to other gram-negative bacterial infections [88]; the mortality rate for patients with a *P. aeruginosa* bloodstream infection has been placed at between 18% and 61% [89].

P. aeruginosa is well known for its highly antibiotic-resistant profile which makes treatment difficult [39, 90]. It is intrinsically resistant to a large number of antibiotics, due to low cell membrane permeability to these antibiotics and the presence of a number of efflux pumps [39, 90, 91]. In addition, it readily evolves resistance to further antibiotics when challenged with them [90, 92-94]. Most clinical isolates have evolved resistance to at least one antibiotic family [4], and strains which have evolved multiple-antibiotic resistance are also common and increasing [7, 8, 90, 92].

As well as its role as an important human pathogen, *P. aeruginosa* is a well-studied model organism and is easily culturable in laboratory conditions. In this thesis, I use the strain PA01, which was isolated from a burn wound in 1954.

1.6 Major objectives of this thesis

In this thesis, I set out to investigate how various multiple-antibiotic treatments affect the rate of evolution of antibiotic resistance in the opportunistic human pathogen *Pseudomonas aeruginosa*. I will consider both molecular mechanisms affecting the evolution of resistance (the interactions between antibiotics in combination) and also the ecological context (antibiotic heterogeneity). In Chapter 2, I will systematically characterise the interactions in 120 antibiotic combinations, and identify trends in the types of interactions present. In Chapter 3, I will take a subset of these antibiotic combinations, and test the influential but weakly-evidenced hypothesis that synergistic combinations accelerate the evolution of antibiotic resistance. In Chapter 4, I will focus

on one antibiotic combination, ceftriaxone - sulfamethoxazole, and test a hypothesis hinted at in my earlier data that this combination may be particularly effective in preventing the evolution of antibiotic resistance. In Chapter 5, I will, for the first time, use an experimental evolution approach to test how varying levels of antibiotic heterogeneity affect the evolution of antibiotic resistance.

Overall I will use a systematic experimental evolution approach, working at a large scale, with the aim to further understanding of the usefulness of multiple-antibiotic treatments.

Chapter 2

2 Characterising interactions between pairs of antibiotics in *Pseudomonas aeruginosa*

2.1 Introduction

In order to address the increasing clinical challenge posed by antibiotic resistant pathogens, multiple-antibiotic combinations are being considered as a promising treatment option [16, 17]. It has been suggested that antibiotic combinations may be more effective at inhibiting bacterial growth than single antibiotic treatments, may increase the chance of at least one effective antibiotic being used, and may hinder the evolution of antibiotic resistance [16, 95]. There is, however, controversy about the effectiveness of multiple-antibiotic combinations in clinical situations [67, 95-102].

An important consideration for clinicians considering multiple-antibiotic combinations is that the antibiotics used may interact with each other, and enhance or reduce each other's inhibitory effects on bacteria. Combinations may interact synergistically, where the inhibitory effect of two antibiotics in combination is greater than would be expected if the two antibiotics acted independently of each other, or antagonistically, where the inhibitory effect of two antibiotics in combination is less than would be expected if the two antibiotics acted independently of each other (see Fig. 1.3). Clinicians tend to favour synergistic combinations due to their enhanced effectiveness at clearing

infection for a given concentration of antibiotics. Antagonistic combinations are generally avoided clinically.

The mechanisms generating synergistic and antagonistic interactions are not fully understood, though a number of explanations have been proposed for particular synergistic or antagonistic interactions. Synergy may occur when two antibiotics inhibit two different targets carrying out the same function [103]. Genetic redundancy may mean that an antibiotic inhibiting one target has limited effect, and the effect is much enhanced when a second target is also inhibited. Antagonism is predicted to occur when the two antibiotics target genes involved in the same metabolic pathway, as the productivity of the pathway will be limited by the least productive step, and additional inhibition in other parts of the pathway may be of limited importance to bacterial fitness. MacLean demonstrates this in an experiment finding an antagonistic interaction between rifampicin and streptomycin, in a *Pseudomonas aeruginosa* system [104]. Both rifampicin and streptomycin target protein synthesis: rifampicin inhibits RNA polymerase and streptomycin, an aminoglycoside antibiotic, interferes with ribosomal function. Bollenbach *et al.* suggest that antagonism may occur when microbes respond in a way that is non-optimal for microbial growth rate to the presence of one antibiotic, and the addition of a second antibiotic can actually improve the bacterial response to the first antibiotic [105]. Specifically, an antagonistic interaction between DNA synthesis inhibitors and protein synthesis inhibitors can be explained as bacterial cells over-express ribosomes in the presence of DNA stress, leading to an inefficient use of resources and slower than optimal growth. Adding a protein synthesis inhibitor can reduce ribosomal expression to nearer the optimum level under conditions of DNA stress.

Antibiotics can inhibit bacteria through a bactericidal or a bacteriostatic effect. Bactericidal antibiotics kill bacteria, whereas bacteriostatic antibiotics inhibit growth of bacteria but do not kill them so bacterial growth may resume when the antibiotic concentration declines. (Further discussion of antibiotics that are generally seen as bactericidal and bacteriostatic, and the limitations of these classifications, can be found on p6.) Previous work has suggested that combinations involving two bactericidal antibiotics may often be synergistic, whereas those involving a bactericidal and a bacteriostatic antibiotic may be more likely to produce an antagonistic interaction [30, 106]. It has been suggested that bactericidal antibiotics have a common killing mechanism whereby they induce changes in bacterial gene expression which result in oxidative stress and cell death [107]. If this hypothesis is correct, where the rate of gene expression is slowed by a bacteriostatic antibiotic, we would expect to see a protective effect of the bacteriostatic antibiotic against a bactericidal antibiotic.

The characterisation of interactions between antibiotics is of interest in elucidating the mechanisms by which bacteria respond to antibiotics [16, 23] and more generally how bacterial cells respond to perturbations [108]. Interactions can provide insight into epistasis between mutations; antibiotics are used to inhibit specific genes, mimicking the effect of mutations in those genes, and the effect on bacterial growth rate is quantified [104]. Most studies have characterised the interactions between a small number of antibiotics (see for example [30, 109-111]); relatively few studies have systematically characterised antibiotic interactions for particular bacterial species. Systematic studies allow trends to be identified in the type of interactions shown. For example, a study characterising all pairwise interactions amongst a set of antibiotics in *Escherichia coli* found that the antibiotics could be grouped into monochromatically

interacting classes, so that two classes interacted only synergistically or only antagonistically [108]. These classes corresponded to the mechanism of action of the antibiotics. In addition, systematic studies may highlight new combinations which appear to show useful properties for the clinic or laboratory [112].

In this work, we systematically characterise the two-way interactions among a set of sixteen antibiotics (a total of 120 antibiotic pairs), in a *P. aeruginosa* experimental system. As well as allowing us to identify trends in the types of interactions shown, we plan to use the results to select a set of antibiotic pairs with a range of interactions to test the recently-proposed hypothesis that synergistic interactions accelerate the evolution of antibiotic resistance [68, 113] (see Chapter 3).

2.2 Materials and Methods

2.2.1 Conditions, strain and antibiotics used

All experiments were conducted in Mueller-Hinton 2 liquid media (Fluka 90922), with bacteria grown at 37°C. The populations were founded from a single clone of luminescent-tagged *P. aeruginosa* PA01: mini-Tn7T-Gm-*lux*. This strain carries a bioluminescent reporter construct (the *lux* construct) that allows rapid and sensitive measurements of bacterial growth rate across a broad dynamic range (see [108]).

The reaction producing luminescence consumes a small proportion of cellular ATP. It has been suggested that lower intracellular levels of ATP may reduce bacterial mutation

rates [114], which could delay the evolution of antibiotic resistance by reducing the supply of resistance-conferring mutations. We do not have experimental data to directly address this point. However, we concluded that the supply of resistance-conferring mutations did not limit the rate of evolution of resistance in the work conducted in Chapters 3 and 4 of this thesis. The results presented in Chapter 5 do not provide a clear indication as to whether mutation rate was limiting the rate of evolution of resistance in this experiment. Please see Chapter 6 for further discussion of this issue.

Sixteen antibiotics were selected for use in this experiment, with the aim to capture a broad range of mechanisms of action and to select antibiotics that are suitable for use in the lab. Antibiotic solutions were made from powder stocks as indicated in Table 2.1. All possible antibiotic pairs using this set of sixteen antibiotics were tested; a total of 120 antibiotic combinations. Treatments were also set up with each antibiotic singly, as well as an antibiotic-free control treatment. The antibiotic concentrations given in Table 2.1 were selected with the intention that each antibiotic individually would reduce log sensitive bacterial growth rate by between 30% and 50%, but would not completely inhibit growth of sensitive bacteria. In practice, there was a wider variation in the inhibitory effect of each antibiotic individually.

Note that the region of decline in bacterial growth rate, from growing as quickly as with no antibiotics present, to no growth, is often roughly linear. However in some cases the decline is a different function. My experiments were designed so as not to assume a linear dose-response curve. In Chapter 4 where I used multiple concentrations of the same antibiotic, I selected the antibiotic concentrations to use based on their inhibitory

effect, in order to select concentrations that produced the desired degree of inhibition regardless of the shape of the dose-response curve. In the other data chapters only one concentration was used for each antibiotic.

Antibiotic	**Manufacturer code**	**Concentration used (mg/l)**	**Molarity (M)**	**Antibiotic family**
Cefsulodin	C8145 (Sigma)	17	0.000031 (anhydrous basis)	B-lactams
Cefotaxime	C7039 (Sigma)	140	0.00029	B-lactams
Ceftriaxone	C5793 (Sigma)	3.9	0.0000059	B-lactams
Piperacillin	P8396 (Sigma)	14	0.000026	B-lactams
Meropenem	M2574 (Sigma)	0.47	0.0000011	B-lactams
Carbenicillin	C3416 (Sigma)	50	0.00012	B-lactams
Polymyxin B	P1004 (Sigma)	0.60	0.00000043	Polymyxins
Kanamycin	K1876 (Sigma)	110	Molecular mass of K1876 (Sigma) not available	Aminoglycosides
Tobramycin	T1783 (Sigma)	0.41	0.00000088 (free base basis)	Aminoglycosides
Amikacin	A1174 (Sigma)	0.16	Molecular mass of	Aminoglycosides

			A1174 (Sigma) not available	
Ciprofloxacin	17850 (Sigma)	0.18	0.00000054	Quinolones
Enoxacin	E3764 (Sigma)	0.13	0.00000041	Quinolones
Levofloxacin	28266 (Sigma)	0.09	0.00000025	Quinolones
Mafenide	A2134 (Sigma)	150	0.00067	Sulphonamides
Sulfamethoxazole	S7507 (Sigma)	530	0.00209	Sulphonamides
Rifampicin	R3501 (Sigma)	6.8	0.0000083	Rifamycins

Table 2.1. Antibiotics used in the experiments described here, with manufacturer code, concentration bacteria were exposed to (either as a single antibiotic or as part of a combination) in mg/l, molarity in M (where available), and the family each antibiotic belongs to.

2.2.2 Experimental work

To form the populations to be exposed to each antibiotic, first a bacterial culture was grown from freezer stocks to saturation in antibiotic free conditions. Bacteria were then diluted to allow approximately 10^5 cells per well, into media containing an antibiotic combination, an individual antibiotic or no antibiotic depending on the treatment.

Measurements were taken by culturing populations in static conditions and assaying luminescence expression every 20 minutes for 24 hours using a spectrometer (FLUOStar OPTIMA, BMG Labtech). All plate set-ups and transfers were carried out using an automated pipettor (Precision XS, BioTek).

On each experimental run, there were two replicates of each combination, six of each antibiotic individually and 32 replicates of the antibiotic-free control. Four separate experimental runs were carried out. The plates used were black 384-well plates (black plates were used in order to minimise cross-talk between wells). The culture volume in each well was 80μl. Please see Appendix B for the layout of the treatments within each 384 well plate- this layout was chosen to reduce the time taken to setup the experimental plate (which was still approximately 90 minutes due to the number of different antibiotics included in the experiment). The FLUOStar OPTIMA spectrometer read plates in the pattern indicated in Appendix B. The Precision XS automated pipettor multi-channel function was used to transfer 8 cultures at a time during plate transfers, as indicated in Appendix B.

2.2.3 Calculation of the degree of synergy (S) for each combination

In this work, we refer to the type and degree of interaction as the degree of synergy, S. We used the Bliss independence model as our null model for calculation of S. The Bliss independence model assumes that there is no interaction between antibiotics in a combination (so the interaction is additive); for example if antibiotic A and antibiotic B each reduce bacterial growth by 50%, the growth rate of bacteria exposed to antibiotics

A and B in combination under the Bliss model will be: 0.5 x 0.5 = 0.25, or a 75% reduction in growth rate [115]. Interactions are characterised as synergistic where the growth rate of bacteria exposed to two antibiotics in combination is less than predicted by the growth rate of bacteria exposed to each antibiotic separately, and as antagonistic where the growth rate of bacteria exposed to two antibiotics in combination is greater than predicted by the growth rate of bacteria exposed to each antibiotic separately (see Fig. 1.3).

In accordance with the method of Hegreness *et al*. [68] for determining interaction values for antibiotic combinations, the exponential growth rate of bacteria exposed to each combination and individual antibiotic treatment was calculated and used for determination of degree of synergy. Log_{10} luminescence - time curves were plotted for each population, and exponential growth rate calculated as the steepest gradient over a five hour window (see Appendix C for further details on the calculation of exponential growth rate). The degree of synergy, S, for each combination on each experimental run was calculated as S = (p_{Ab}/p_{ab}) (p_{aB}/p_{ab}) - (p_{AB}/p_{ab}) where p_{Ab} is the mean exponential growth rate of bacteria exposed to antibiotic A only, p_{aB} is the mean exponential growth rate of bacteria exposed to antibiotic B only, p_{AB} is the mean exponential growth rate of bacteria exposed to antibiotics A and B in combination, and p_{ab} is the mean exponential growth rate of a control grown in antibiotic-free conditions. An overall mean for each antibiotic combination across the experimental runs was then calculated.

2.2.4 Statistical analyses

We tested the hypothesis that S was different from 0 (i.e. there was an interaction between the antibiotics) for each combination, using t-tests. As we performed 120 t-tests, it is likely we found some false-positive results. To correct for this we used the false discovery rate control procedure proposed by Verhoeven *et al.* [116].

To test whether the two antibiotic families that are interacting (the 'block' in Table 2.2) affect the degree of synergy, we coded each block with a letter and ran an ANOVA analysis. As block did affect the degree of synergy, for the remaining analyses we averaged across antibiotics within a block to produce one data point per block. To test whether bactericidal and bacteriostatic antibiotics produced different degrees of synergy, we coded whether a combination involved two bactericidal antibiotics, two bacteriostatic antibiotics or a bactericidal and a bacteriostatic antibiotic, and ran an ANOVA analysis.

2.3 Results

We characterised all two-way interactions between a set of 16 antibiotics; 120 antibiotic combinations in total. From these 120 combinations, we identified 41 combinations which showed a significantly synergistic interaction and 11 which showed a significantly antagonistic interaction (Fig. 2.1 and Table 2.2, see Materials and Methods for an explanation of how significance was determined).

The antibiotic family a particular antibiotic is a member of may give some information about the degree of synergy the antibiotic is likely to show, as antibiotics within a family share similar mechanisms of action. Consistent with this hypothesis, we found that block (i.e. a term coding the two antibiotic families that are interacting, as indicated in Fig. 2.1 / Table 2.2) had an effect on the degree of synergy ($F_{18, 101} = 6.30$, $P < 0.001$). This was an overall F test. Therefore, for our final analysis we averaged across the antibiotics within a family to produce one data point per block.

Antibiotic family		**B-lactam**						**Polymyxin**	**Aminoglycoside**			**Quinolone**			**Sulphonamide**		**Rifa-mycin**
	Antibiotic name	*Cefsul-odin*	*Cefotax-ime*	*Ceftri-axone*	*Piper-acillin*	*Merope-nem*	*Carben-icillin*	*Polymyxin B*	*Kana-mycin*	*Tobra-mycin*	*Amikacin*	*Ciprofl-oxacin*	*Enox-acin*	*Levoflo-xacin*	*Mafeni-de*	*Sulfameth-oxazole*	*Rifamp-icin*
B-lactam	*Cefsulodin*																
	Cefotaxime	0.129															
	Ceftriaxone	0.068	0.004														
	Piperacillin	**0.144**	0.259	0.183													
	Meropenem	**0.095**	0.045	0.050	**0.863**												
	Carbenicillin	**0.159**	0.166	0.170	**0.290**	0.275											
Polymyxin	*Polymyxin B*	**0.254**	-0.156	**0.252**	**0.369**	**0.369**	**0.203**										
Amino-glycoside	*Kanamycin*	**0.271**	0.112	**0.212**	**0.324**	**0.290**	**0.238**	**0.347**									
	Tobramycin	**0.127**	0.277	**0.273**	**0.614**	**0.455**	**0.357**	**0.414**	**0.476**								
	Amikacin	0.044	0.155	0.262	**0.504**	**0.351**	**0.206**	**0.347**	**0.224**	**0.261**							
Quinolone	*Ciprofloxacin*	0.035	-0.136	**0.306**	0.147	**0.273**	**0.264**	**0.179**	**0.091**	0.043	**-0.099**						
	Enoxacin	0.108	0.221	0.177	**0.237**	0.107	0.202	**0.228**	**0.217**	0.147	0.039	0.101					
	Levofloxacin	0.009	0.060	**0.176**	**0.184**	0.151	0.202	0.108	-0.022	0.014	-0.107	0.054	0.068				
Sulphona-mide	*Mafenide*	-0.128	0.207	0.096	0.167	0.217	0.260	**0.162**	0.053	**-0.168**	-0.052	0.010	0.208	**-0.259**			
	Sulfamethoxa-zole	-0.110	-0.075	**-0.093**	-0.011	0.058	-0.050	-0.175	-0.221	-0.073	**-0.192**	-0.123	**-0.309**	**-0.280**	**-0.203**		
Rifamycin	*Rifampicin*	-0.006	0.037	0.089	0.058	-0.096	0.064	0.125	0.040	**-0.116**	**-0.121**	-0.057	-0.100	-0.051	-0.115	**-0.142**	

Figure 2.1A. The type of interaction observed for each of the 120 possible pairwise combinations between 16 antibiotics. Bold indicates a statistically significant interaction. Red indicates a synergistic interaction, with a darker shade representing a stronger interaction. Blue indicates an antagonistic interaction, with a darker shade representing a stronger interaction. Numbers in cells are S values. Some combinations of antibiotic families tended to have more synergistic or more antagonistic interactions than others; S varies with block (blocks separated by bold black lines). See overleaf for key to shading.

Key to shading in Fig. 2.1A

Shading colour	Degree of synergy
	Greater than 0.4
	0.3 - 0.4
	0.2 - 0.3
	0.1 – 0.2
	0 - 0.1
	-0.1 – 0
	-0.2 - -0.1
	-0.3 - -0.2
	-0.4 - -0.3

Antibiotic family		**B-lactam**						**Polymyxin**	**Aminoglycoside**			**Quinolone**			**Sulphonamide**		**Rifamycin**
	Antibiotic name	*Cefsulodin*	*Cefotaxime*	*Ceftriaxone*	*Piperacillin*	*Meropenem*	*Carbenicillin*	*Polymyxin B*	*Kanamycin*	*Tobramycin*	*Amikacin*	*Ciprofloxacin*	*Enoxacin*	*Levofloxacin*	*Mafenide*	*Sulfamethoxazole*	*Rifampicin*
B-lactam	*Cefsulodin*																
	Cefotaxime	0.048															
	Ceftriaxone	0.024	0.085														
	Piperacillin	0.025	0.134	0.068													
	Meropenem	0.009	0.073	0.052	0.043												
	Carbenicillin	0.011	0.097	0.062	0.039	0.106											
Polymyxin	*Polymyxin B*	0.052	0.148	0.042	0.061	0.040	0.043										
Aminoglycoside	*Kanamycin*	0.038	0.112	0.040	0.052	0.041	0.022	0.070									
	Tobramycin	0.026	0.066	0.061	0.061	0.063	0.071	0.073	0.047								
	Amikacin	0.018	0.099	0.069	0.047	0.034	0.027	0.020	0.039	0.050							
Quinolone	*Ciprofloxacin*	0.025	0.092	0.020	0.048	0.030	0.006	0.036	0.004	0.038	0.013						
	Enoxacin	0.079	0.056	0.050	0.009	0.032	0.066	0.020	0.034	0.063	0.014	0.009					
	Levofloxacin	0.013	0.074	0.039	0.036	0.112	0.075	0.038	0.020	0.030	0.032	0.052	0.063				
Sulphonamide	*Mafenide*	0.041	0.105	0.062	0.072	0.056	0.070	0.029	0.054	0.028	0.039	0.032	0.088	0.026			
	Sulfamethoxazole	0.049	0.077	0.020	0.089	0.045	0.052	0.065	0.103	0.082	0.027	0.050	0.029	0.045	0.037		
Rifamycin	*Rifampicin*	0.012	0.041	0.057	0.032	0.038	0.039	0.086	0.046	0.022	0.022	0.035	0.029	0.020	0.036	0.029	

Figure 2.1B. Standard errors on the S values given in Figure 2.1A.

Block	**Proportion of synergistic interactions**	**Proportion of antagonistic interactions**
B-lactam / β-lactam	33%	0%
B-lactam / polymyxin	80%	0%
B-lactam / aminoglycoside	72%	0%
B-lactam / quinolone	33%	0%
B-lactam / sulphonamide	0%	8%
B-lactam / rifamycin	0%	0%
Polymyxin / aminoglycoside	100%	0%
Polymyxin / quinolone	67%	0%
Polymyxin / sulphonamide	50%	0%
Polymyxin / rifamycin	0%	0%
Aminoglycoside / aminoglycoside	100%	0%
Aminoglycoside / quinolone	22%	11%
Aminoglycoside / sulphonamide	0%	33%
Aminoglycoside / rifamycin	0%	67%
Quinolone / quinolone	33%	0%
Quinolone / sulphonamide	0%	50%
Quinolone / rifamycin	0%	0%
Sulphonamide / sulphonamide	0%	100%
Sulphonamide / rifamycin	0%	50%

Table 2.2. The proportion of significantly synergistic and significantly antagonistic interactions for each antibiotic family combination used.

Note that there are some discrepancies between the degree of synergy obtained in the Chapter 2 experiments and the Chapter 3 experiments for the interaction of the same pair of antibiotics. I don't have a definite answer as to why this is, but possible explanations include the fact that different concentrations of antibiotics were used, as degree of synergy is known to vary with antibiotic concentration [**16, 30, 122**]. Differences in laboratory conditions, for example humidity, between different days may also have affected results. There were significant issues with variations in the growth

rate of bacteria exposed to the same concentration of an antibiotic on different days during the Chapter 2 and 3 experiments, and this issue may have affected the degrees of synergy obtained for particular antibiotic pairs.

Bactericidal antibiotics kill bacteria, whereas bacteriostatic antibiotics inhibit bacterial growth without killing the cells. Out of the antibiotic families we used, the β-lactams, polymyxins, aminoglycosides and quinolones are bactericidal, whereas the sulphonamides and rifamycins are bacteriostatic. We found that whether the antibiotics interacting were bactericidal or bacteriostatic influenced the degree of synergy ($F_{2,\ 16}$ = 17.9, $P < 0.001$). This was an overall F test. Interactions involving two bactericidal antibiotics tended to be synergistic, interactions involving a bactericidal and a bacteriostatic antibiotic close to additive, and interactions involving two bacteriostatic antibiotics antagonistic (Fig. 2.2).

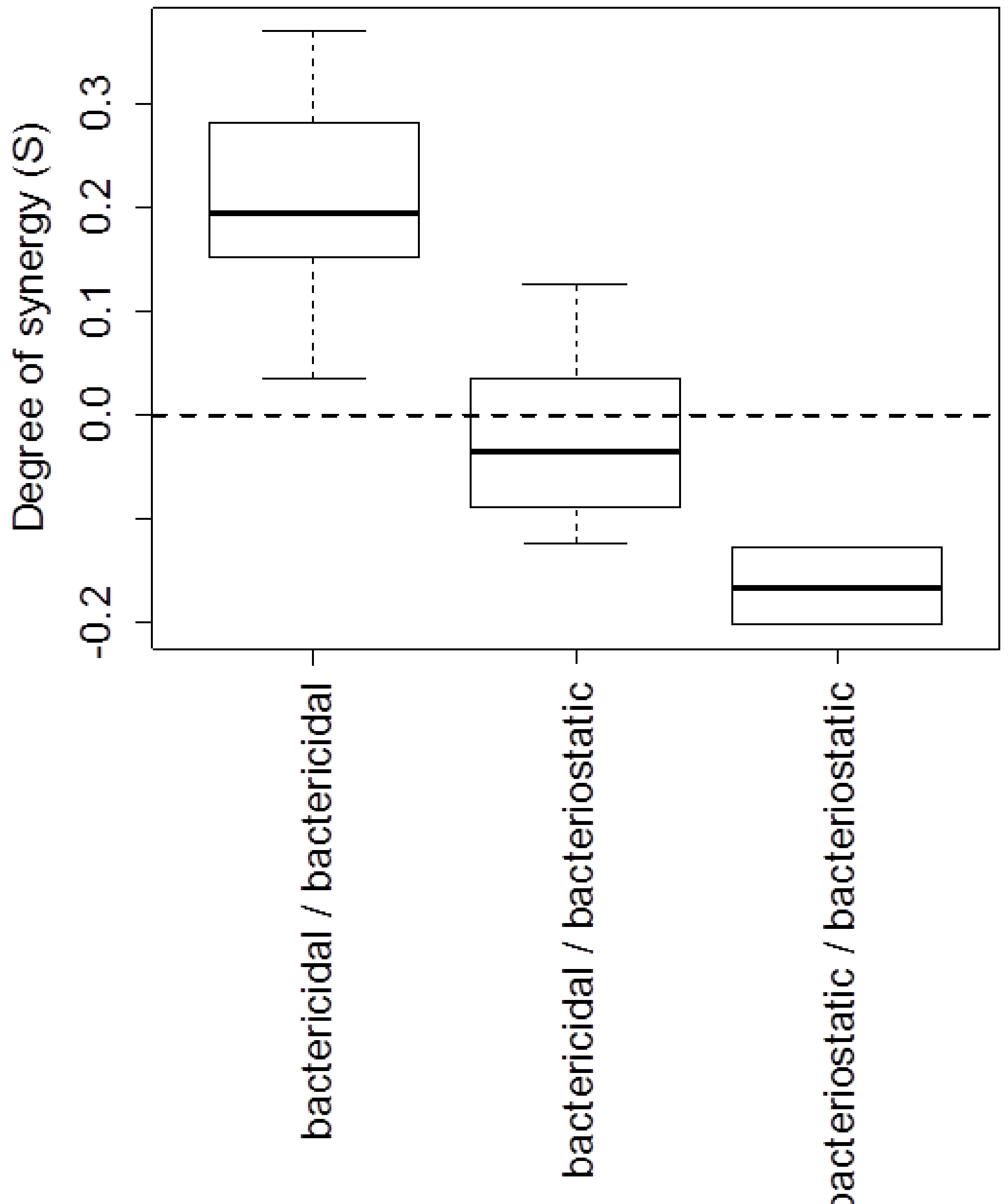


Figure 2.2. The mechanism of action of antibiotics in combination affects the degree of synergy. Bactericidal / bactericidal combinations tend to show synergistic interactions, bactericidal / bacteriostatic combinations near additive interactions, and bacteriostatic / bacteriostatic combinations antagonistic interactions. S > 0 represents synergy and S < 0 antagonism. The dotted line represents S = 0 where interactions are additive.

2.4 Discussion

In this work, we systematically characterise the interactions between antibiotics in 120 antibiotic pairs. We identify a number of synergistic and antagonistic interactions, and determine that antibiotics within a particular antibiotic family tend to show similar interactions to each other (see Fig. 2.1). In addition, we find that interactions are more synergistic when two bactericidal antibiotics interact, and more antagonistic when two bacteriostatic antibiotics interact (see Fig. 2.2).

A previous study of clinical isolates of multiple-antibiotic resistant *P. aeruginosa* found that synergy is most common in β-lactam / quinolone combinations, and that some β-lactam / aminoglycoside combinations were also synergistic [117]. We also find that β-lactam / quinolone and β-lactam / aminoglycoside combinations are often synergistic. Additionally, we find that combinations involving polymyxins are often synergistic, supporting the previously suggested hypothesis that polymyxins may enhance the action of other antibiotics by increasing cell permeability to other antibiotics [118].

In their *E. coli* study, Yeh *et al*. find that the interactions antibiotics display can be predicted by the cellular function the interacting antibiotics each target [108]. Consistent with this result, we find that the antibiotic families which are interacting have an effect on the degree of synergy; it is possible to predict whether an interaction will be more or less synergistic than average based on the antibiotic families involved. An interesting outcome from our work is the difference in the degree of synergy shown by bactericidal and bacteriostatic antibiotics, which supports previous work suggesting that bactericidal / bacteriostatic combinations show less synergy than bactericidal /

bactericidal combinations [30]. However, I did not find that bactericidal / bacteriostatic combinations showed antagonistic interactions, in contrast to suggestions that bactericidal antibiotics have a common killing mechanism which bacteriostatic antibiotics can protect against [107].

The degree of synergy obtained experimentally can be influenced by a number of factors; for example the inoculum size, variation between strains and the media in which testing is carried out may influence the results [16, 119, 120]. In addition, degree of synergy may change with the concentration of antibiotics used [16, 30, 121], as may the bactericidal or bacteriostatic properties of an antibiotic [23, 30, 122]. Different authors have used different methods for quantifying synergy and antagonism, making comparison between studies difficult (see for example [16, 123, 124]). The interactions we have quantified may therefore only be applicable to our experimental system, although we believe our results regarding consistent interaction types within antibiotic families, and the effect of bactericidal and bacteriostatic antibiotics on the degree of synergy, may be more generally applicable given their consistency with other studies. Based on our results, we decided to use one antibiotic from each antibiotic family for our experimental evolution study described in Chapter 3; this provided a set of six antibiotics forming a total of 15 antibiotic pairs.

Chapter 3

3 Effect of interactions between antibiotics on the rate of evolution of resistance in *Pseudomonas aeruginosa*

3.1 Introduction

Antibiotic resistance is a growing clinical burden and is likely to be a key public health challenge in coming decades [7, 9]. Multiple-antibiotic combinations are increasingly being considered as a promising treatment option to combat antibiotic-resistant pathogens or pathogens prone to evolving resistance [16, 17]. Antibiotic combinations may offer more complete inhibition of bacteria than use of a single antibiotic on its own, maintain effective treatment even when bacteria are resistant to one of the antibiotics in a combination, or prevent the evolution of antibiotic resistance [16, 95]. The use of such combinations in clinical settings is however, controversial, with mixed evidence as to the success of combination therapy in reducing mortality or preventing the evolution of resistance compared to single antibiotic therapy [67, 95-102, 125, 126].

A potentially useful property of multiple-antibiotic combinations is that the antibiotics may interact with each other and alter each other’s effects on bacterial growth and survival. Combinations may interact synergistically, where the inhibitory effect of two antibiotics in combination is greater than would be expected if the two antibiotics acted

independently of each other, or antagonistically, where the inhibitory effect of two antibiotics in combination is less than would be expected if the two antibiotics acted independently of each other (see Fig. 1.3). Synergistic combinations are generally favoured clinically as such combinations are more effective than a non-interacting combination in inhibiting bacterial growth and clearing infection for a given concentration of antibiotics. However, two recent studies suggested that synergistic combinations, while more effective at inhibiting bacteria, may accelerate the evolution of antibiotic resistance. In contrast antagonistic combinations, while less effective at inhibiting bacterial growth, may slow or even prevent the evolution of antibiotic resistance [68, 113].

Antibiotic resistant mutants will spread in a population when the resistant mutants have a selective advantage over the antibiotic-sensitive wild type strain (for example, resistant mutants may grow more quickly in the presence of antibiotics than the sensitive strain). How quickly resistant mutants spread is determined by the size of the selective advantage of the resistant mutants over the sensitive strain; the higher the growth rate of the resistant mutants compared to the sensitive wild type strain, the quicker they increase in frequency. Interactions between antibiotics in combination may change the selective advantage of resistant mutants by altering either the growth rate of the sensitive wild type strain, or the growth rate of the resistant mutants. Synergistic combinations are, by definition, more inhibitory to the sensitive wild type strain for a given concentration of antibiotics than antagonistic combinations. By increasing the selection pressure for resistance, synergistic combinations might be expected to accelerate the evolution of resistance. In this paper, we refer to the type and degree of interaction between antibiotics in combination as the ‘degree of synergy’, and the effect

of degree of synergy on the growth rate of the sensitive wild type strain as the ‘effect on sensitive strain’.

In addition to the effect on the sensitive strain, Hegreness and co-workers have proposed that the degree of synergy can affect the growth rate of resistant mutants even if a synergistic and antagonistic combination have identical inhibitory effects on the sensitive strain [68] (see Fig. 3.1). This is because of the differential effects of synergistic and antagonistic combinations on mutants fully or partially resistant to one antibiotic in the combination. Hegreness *et al.* suggest that exposed to a synergistic combination, mutants resistant to one antibiotic overcome at least part of the inhibitory effect of that antibiotic, and also part of the synergistic effect of both antibiotics working in combination, so the resistant mutants have a large selective advantage over the sensitive strain. Exposed to an antagonistic combination, mutants resistant to one antibiotic overcome at least part of the inhibitory effect of that antibiotic, but ‘unmask’ more of the inhibitory effect of the second antibiotic by removing part of the protective effect of the first antibiotic against the second antibiotic. Thus Hegreness *et al.* propose that the singly-resistant mutant enjoys a greater selective advantage over the sensitive strain when exposed to a synergistic rather than an antagonistic combination, and the synergistic combination will accelerate the evolution of resistance. We refer to the effect of degree of synergy on the absolute selective advantage of mutants resistant to one antibiotic in the combination as the ‘effect on singly-resistant mutant’. The ‘effect on singly-resistant mutant’ assumes that resistance to different antibiotics is independent; acquiring a mutation conferring resistance to one antibiotic does not also confer resistance to the second antibiotic in a combination (in other words, there is no cross-resistance).

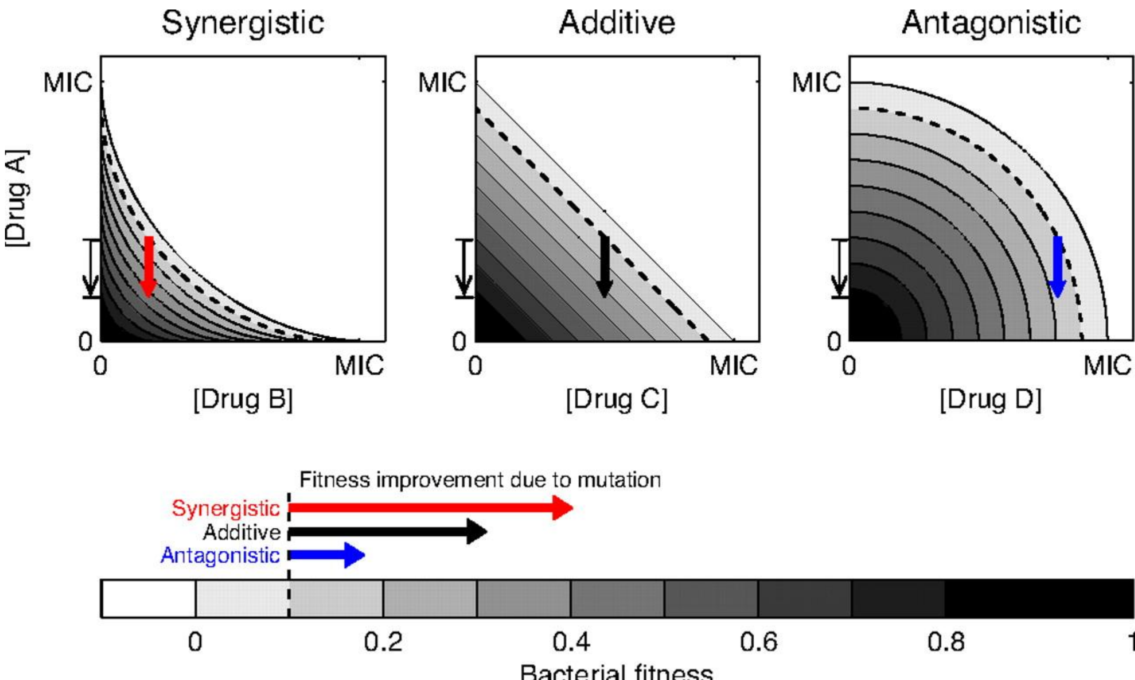


Figure 3.1. Geometric model showing that a mutation conferring resistance to a single antibiotic is most advantageous with a synergistic antibiotic combination. The isoboles represent lines of equal bacterial growth rate, with darker shading representing a higher bacterial growth rate. Antibiotic A may interact with antibiotic B (a synergistic interaction), antibiotic C (an additive interaction), or antibiotic D (an antagonistic interaction). The arrows represent the selective advantage of a single resistance mutation conferring partial resistance to antibiotic A, when in an environment containing synergistically interacting antibiotics (red arrow), additively interaction antibiotics (black arrow), or antagonistically interacting antibiotics (blue arrow). Figure taken with permission from Hegreness, M., et al., *Accelerated evolution of resistance in multidrug environments.* Proceedings of the National Academy of Sciences, 2008. **105**(37): p. 13977-13981. Copyright (2008) National Academy of Sciences, USA.

Note that Figure 3.1, taken from the Hegreness *et al.* 2008 paper [68], is based on the Loewe additivity null-model of interactions between antibiotics. However, the calculations of degree of synergy in the Hegreness paper were based on the Bliss independence null-model, as was the calculation of degree of synergy in my experiments. (See p20-21 for a comparison of the Loewe additivity and Bliss independence null-models.) This is an inconsistency in the Hegreness paper.

Theory predicts that synergistic interactions should accelerate the evolution of antibiotic resistance compared to antagonistic interactions, because of the effect on the sensitive strain and on the singly-resistant mutant. However, we should also consider how interactions between antibiotics might affect the supply of resistance-conferring mutations to a population, before selection can act to change the frequency of these mutations. The rate of supply of mutations to a population is the product of the intrinsic mutation rate and the population size, so that smaller populations have a lower mutation supply rate. Synergistic combinations are more effective at reducing population size than antagonistic combinations, and so we might expect synergistic interactions to reduce the rate of evolution of resistance through this mechanism. The relative importance of synergistic interactions reducing the supply of resistance-conferring mutations and increasing the selective advantage of these mutations once they arise on the evolution of resistance is unclear.

Here, we conduct experiments to assess the impact of antibiotic interactions on the evolution of resistance in the important opportunistic human pathogen *Pseudomonas aeruginosa*, using experimental evolution. Multiple-antibiotic resistance is a particular clinical problem in *P. aeruginosa* [8, 90, 127, 128], and combination therapy is often used for severe infections [102, 128]. We experimentally challenge *P. aeruginosa* with adapting to combinations of two antibiotics composed from a set of antibiotics with representatives of all the major families used against *P. aeruginosa* clinically (15 combinations in total). Using this approach, we find that antibiotic interactions do not alter the rate of evolution of resistance. Instead, we find that the rate of evolution of resistance correlates strongly to the extent of antibiotic-mediated inhibition of growth for both single antibiotics and two-antibiotic combinations.

3.2 Materials and Methods

3.2.1 Conditions, strain and antibiotics used

All experiments were conducted in Mueller-Hinton 2 liquid media (Fluka 90922), with bacteria grown at 37°C. The populations were founded from a single clone of luminescent-tagged *P. aeruginosa* PA01: mini-Tn7T-Gm-*lux*. This strain carries a bioluminescent reporter construct (the *lux* construct) that allows rapid and sensitive measurements of bacterial growth rate across a broad dynamic range (see [108]). All plate set-ups and transfers were carried out using an automated pipettor (Precision XS, BioTek).

Antibiotic solutions were made from powder stocks as indicated in Table 3.1. The antibiotics used in these experiments cover the range of mechanisms of action found in the major classes of antibiotics used against *P. aeruginosa* clinically. Ceftriaxone, ceftazidime, cefotaxime, piperacillin and carbenicillin are β-lactam antibiotics; they bind penicillin-binding proteins and prevent normal cross-linking of peptides in the bacterial cell wall. Amikacin, kanamycin and tobramycin are aminoglycoside antibiotics; they work by inhibiting protein synthesis. Levofloxacin, sparfloxacin, ciprofloxacin and pefloxacin are fluoroquinolone antibiotics; they inhibit the action of DNA gyrase [21]. Polymyxin B is a cationic surface-active compound which is thought to disrupt membrane organisation [129]. Sulfamethoxazole is a member of the sulphonamide family of antibiotics and inhibits folate synthesis [130]. Rifampicin binds to the β-subunit of RNA polymerase and blocks transcription [29]. For the selection experiment, one antibiotic was chosen as a representative from each antibiotic family; these antibiotics were ceftriaxone, amikacin, levofloxacin, polymyxin B,

sulfamethoxazole and rifampicin. All possible combinations of two antibiotics using this set of six antibiotics were created; a total of 15 combinations. In addition, treatments were set up using each of these antibiotics individually, and also the additional antibiotics listed in Table 3.1 individually for use in the cross-resistance assays. The antibiotic concentrations given in Table 3.1 were selected with the intention that each antibiotic individually would reduce log sensitive bacterial growth rate by between 30% and 50%, but would not completely inhibit growth of sensitive bacteria. Some antibiotic concentrations were altered from concentrations used in Chapter 2, with the aim of achieving the correct level of reduction in bacterial growth rate and based on additional pilot work. In practice, there was a wider variation in the inhibitory effect of each antibiotic individually.

Antibiotic	**Manufacturer code**	**Concentration used (mg/l)**	**Molarity (M)**	**Antibiotic family**
Cefotaxime	C7039 (Sigma)	160	0.00034	B-lactams
Ceftriaxone	C5793 (Sigma)	4.5	0.0000068	B-lactams
Piperacillin	P8396 (Sigma)	17	0.000032	B-lactams
Carbenicillin	C3416 (Sigma)	57	0.00013	B-lactams
Polymyxin B	P1004 (Sigma)	0.17	0.00000012	Polymyxins
Kanamycin	K1876 (Sigma)	130	Molecular mass of K1876 (Sigma) not available	Aminoglycosides
Tobramycin	T1783	0.47	0.0000010 (free base	Aminoglycosides

	(Sigma)		basis)	
Amikacin	A1174 (Sigma)	0.19	Molecular mass of A1174 (Sigma) not available	Aminoglycosides
Ciprofloxacin	17850 (Sigma)	0.20	0.00000060	Quinolones
Levofloxacin	28266 (Sigma)	0.10	0.00000028	Quinolones
Sulfamethoxazole	S7507 (Sigma)	600	0.0024	Sulphonamides
Rifampicin	R3501 (Sigma)	7.8	0.0000095	Rifamycins
Ceftazidime	C3809 (Sigma)	23	0.000042	B-lactams
Sparfloxacin	56968 (Sigma)	1.7	0.0000043	Quinolones
Pefloxacin	P0106 (Sigma)	0.24	0.00000052	Quinolones

Table 3.1. Antibiotics used in the experiments described here, with manufacturer code, concentration bacteria were exposed to (either as a single antibiotic or as part of a combination) in mg/l, the molarity in M, and the family each antibiotic belongs to.

3.2.2 Determination of degree of synergy for each antibiotic combination

We used the Bliss independence model as our null model. The Bliss independence model assumes that there is no interaction between antibiotics in a combination (so the interaction is additive); for example if antibiotic A and antibiotic B each reduce bacterial growth rate by 50%, the growth rate of bacteria exposed to antibiotics A and B in combination under the Bliss model will be: 0.5 x 0.5 = 0.25, or a 75% reduction in growth rate [115]. Interactions are characterised as synergistic where the growth rate of bacteria exposed to two antibiotics in combination is less than predicted by the growth rate of bacteria exposed to each antibiotic separately, and as antagonistic where the

growth rate of bacteria exposed to two antibiotics in combination is greater than predicted by the growth rate of bacteria exposed to each antibiotic separately (see Fig. 1.3).

To form the populations to be exposed to each antibiotic, a saturated bacterial culture was diluted into wells of a 384 well plate such that each well contained approximately 100 founding cells. These small founding populations allowed for unique mutants in each population, so that different resistance mutations could be included in the experiment, rather than the same resistance mutations being replicated in every population. If, by contrast, large founding populations were used in each well, there would be a chance that a resistance mutation that had arisen once during the growth of the saturated bacterial culture would be present in many of the founding populations, and would appear as a common resistance mutation, even though it had only actually arisen once. As the antibiotic concentrations used would be lethal to populations of this size, populations were then allowed to grow to saturation in antibiotic-free media before being diluted to allow approximately 10^5 cells per well, into media containing an antibiotic combination, an individual antibiotic or no antibiotic depending on the treatment. Populations were then pre-conditioned to the antibiotics they were to evolve with for 24 hours, before measurements began. Measurements were taken by culturing populations in static conditions and assaying luminescence expression every 20 minutes for 24 hours using a spectrometer (FLUOStar OPTIMA, BMG Labtech). 18 replicate populations were measured for each antibiotic combination and 14 replicate populations for each individual antibiotic treatment, on each of five experimental runs carried out on different days. The final density of each population was also measured using BacTiter-Glo reagent (Promega) at the end of the 24 hour measurement period, and populations

which did not show BacTiter-Glo (Promega) values above those of control wells containing media only were excluded. BacTiter-Glo (Promega) lyses cells and generates a luminescent signal proportional to the amount of ATP present, and within the sensitive range can be used as a proxy for the number of viable cells present.

In accordance with the method of Hegreness *et al*. [68] for determining interaction values for antibiotic combinations, the exponential growth rate of sensitive wild type bacteria when first exposed to each combination and individual antibiotic treatment was calculated and used for determination of degree of synergy. Log_{10} luminescence - time curves were plotted for each population and exponential growth rate calculated as the steepest gradient over a five hour window. The degree of synergy, S, for each combination on each experimental run was calculated as $S = (f_{Ab}/f_{ab})\ (f_{aB}/f_{ab}) - (f_{AB}/f_{ab})$ where f_{Ab} is the mean exponential growth rate of bacteria exposed to antibiotic A only, f_{aB} is the mean exponential growth rate of bacteria exposed to antibiotic B only, f_{AB} is the mean exponential growth rate of bacteria exposed to antibiotics A and B in combination, and f_{ab} is the mean exponential growth rate of a control grown in antibiotic free conditions. An overall average for each antibiotic combination across the experimental runs was then calculated.

3.2.3 Selection experiment

The populations to be exposed to each antibiotic were set up in the same way as for the determination of degree of synergy experiment (see 3.2.2), but using 17 replicate populations for each antibiotic combination, 4 replicates for each individual antibiotic treatment, and 7 replicates of a control grown in antibiotic-free conditions. Instead of

ending the experiment after 24 hours of measurements, the experiment was continued for 9 days. Every 24 hours populations were diluted so that approximately 10^5 cells were transferred to the corresponding treatment on a fresh plate with fresh media. In addition to the luminescence measurements, measurements were taken using BacTiter-Glo reagent (Promega) every two days, taking a sample for measurement just before transfer to fresh media.

The plates used were black 384-well plates (black plates were used in order to minimise cross-talk between wells). The culture volume in each well was 80µl. Please see Appendix B for the layout of the treatments within each 384 well plate- this layout was chosen to simplify plate setup, whilst avoiding introducing bias, for example by consistently placing a treatment at the edge of the plate. The FLUOStar OPTIMA spectrometer read plates in the pattern indicated in Appendix B. The Precision XS automated pipettor multi-channel function was used to transfer 8 cultures at a time during plate transfers, as indicated in Appendix B.

3.2.4 Determination of growth rate of the sensitive ancestral strain and rate of adaptation

In this paper, we refer to the $\log_{10}$ final density measurements produced using Bac-Titer Glo (Promega) as 'growth rate' measurements, as they are a proxy of the number of cell doublings a population has undergone during a 24 hour period (see Appendix A for a validation of the use of Bac-Titer Glo measurements). Growth rate measurements, *f*, were produced for each population at each measurement time. The growth rate of the sensitive ancestral strain (i.e. the bacteria first exposed to antibiotics on Day 1 of the

selection experiment) was calculated as $f_{day1} = f_{AB} / f_{ab}$, where f_{AB} is growth rate of bacteria exposed to a given combination of antibiotics on Day 1 of the selection experiment, and f_{ab} is growth rate of a control grown in antibiotic-free conditions on Day 1. The growth rate of the sensitive ancestral strain is determined by the effect of interactions on the sensitive ancestral strain, plus any differences in the inhibitory effect of the antibiotics applied singly.

To calculate the rate of adaptation, we followed the method proposed by Hegreness *et al*. [68]. First we calculated half of the total adaptation a population achieved over the course of the selection experiment. This was calculated as (0.5 (($f_{AB\ Day9} / f_{ab\ Day1}$) - ($f_{AB\ Day1} / f_{ab\ Day1}$))). We then worked out how long the population took to achieve half the total adaptation. We counted only the time during which a population was actively growing (i.e. in lag or log phases). To calculate the time during each 24 hour period that a population was actively growing, we took the time between the start of luminescence readings on each day and the time at which peak luminescence was reached on that day. We then plotted growth rate at each Bac-Titer Glo measurement point against the cumulative time a population had spent actively growing at that measurement point, using R Version 2.13.2 [131] (see Fig. S2 in Appendix A). Interpolation between the data points was carried out using the *approx* function in order to find the cumulative time a population had been actively growing for when it achieved half the total adaptation. The rate of adaptation was then calculated as half the total adaptation divided by the cumulative time taken to achieve half the total adaptation.

3.2.5 Statistical analyses

All statistical analyses were carried out using R Version 2.13.2 [131]. First, populations which did not show Day 9 growth rate values above those of control wells containing media only were excluded (roughly 25% of populations); most populations excluded had not survived their initial exposure to antibiotics. Mean values of each measure were then calculated for each antibiotic combination. The ceftriaxone - sulfamethoxazole combination was excluded from the analyses because only two populations exposed to this combination had survived. A linear model was fitted to test whether the growth rate of the sensitive ancestral strain, and degree of synergy, affected the rate of adaptation.

To determine whether adaptation progressed differently to combinations of antibiotics than to individual antibiotic environments, we pooled the adaptation data for combinations and individual antibiotics, and used a general linear model to test whether growth rate of the sensitive ancestral strain, and whether an environment contained an individual antibiotic or a combination, affected the rate of adaptation.

3.2.6 Cross-resistance assays

Samples of each population to be assayed were streaked out onto agar plates from freezer stocks frozen down at the end of the selection experiment. After 24 hours of growth, one colony per population was sampled and suspended in antibiotic-free liquid media, to capture one clone per population. Clones were allowed to replicate for 24 hours. Approximately 10^5 cells per population were then introduced into wells containing the assay antibiotic, and preconditioned to the assay environment for 24

hours. After preconditioning, bacteria were diluted so that approximately 10^5 cells were transferred to a fresh corresponding environment. After a further 24 hours of growth, populations were assayed using BacTiter-Glo (Promega). Populations assayed had evolved during the selection experiment in the presence of a single β-lactam, aminoglycoside or quinolone antibiotic listed in Table 3.1. The assay antibiotics were ceftazidime, kanamycin and sparfloxacin, at concentrations of 23 mg/l, 130 mg/l and 1.7 mg/l respectively. All four populations that had evolved with one of the selected antibiotics singly were assayed, and the assay was repeated three times to assay three clones per population.

To analyse the data, first populations that had not grown in antibiotic-free assay conditions were excluded as unviable. Growth rate was calculated as the mean $\log_{10}$ BacTiter-Glo (Promega) value across the clones for each population, and measurements for all populations evolved with the same antibiotic family were then pooled for each assay environment. *t*-tests were carried out to assess whether populations evolved with each antibiotic family had a higher growth rate in each assay environment than the control evolved in antibiotic-free conditions.

3.3 Results

3.3.1 Characterisation of antibiotic interactions in *P. aeruginosa*

The degree of synergy was determined using the Bliss independence model as a null additive model where there are no interactions between antibiotics (see Fig. 1.4 for examples of synergistic and antagonistic combinations). We found a range of

synergistic, antagonistic and near-additive interactions (Fig. 3.2). We have previously found that antibiotics from the same antibiotic family tend to show similar interactions to each other, and so we chose to focus on only a single antibiotic from each family (see Chapter 2).

Antibiotic 1/ Antibiotic 2	**Ceftriaxo-ne**	**Polymyxi-n B**	**Amikacin**	**Levoflox-acin**	**Sulfame-thoxazole**	**Rifa-mpicin**
Ceftriaxone						
Polymyxin B	-0.065 +/- 0.031					
Amikacin	0.031 +/- 0.034	-0.074 +/- 0.013				
Levofloxacin	-0.048 +/- 0.015	-0.126 +/- 0.019	-0.099 +/- 0.015			
Sulfamethoxa-zole	-0.023 +/- 0.059	0.029 +/- 0.036	0.063 +/- 0.037	0.111 +/- 0.034		
Rifampicin	-0.148 +/- 0.045	-0.108 +/- 0.031	-0.050 +/- 0.019	-0.161 +/- 0.013	-0.033 +/- 0.040	

Figure 3.2. Degree of synergy (S) values and standard errors for all 15 antibiotic combinations. S = 0 indicates additivity (no interaction between the two antibiotics in a combination), S < 0 indicates antagonism and S > 0 synergy. Red shading indicates a synergistic interaction, with darker shades representing a stronger interaction. Blue shading indicates an antagonistic interaction, with darker shades representing a stronger interaction. See below for key to shading.

Key to shading in Fig. 3.2

Shading colour	Degree of synergy
	0.1 - 0.2
	0 - 0.1
	-0.1 – 0
	-0.2 - -0.1

3.3.2 Effect of antibiotic interactions on evolution of resistance

To test the hypothesis that synergistic antibiotic combinations accelerate the evolution of antibiotic resistance, we challenged *P. aeruginosa* strain PAO1 with adapting to 15 combinations of antibiotics with a range of interaction types. As controls, we also challenged *P. aeruginosa* with adapting to each antibiotic on its own, and we allowed control populations to evolve in the absence of antibiotics.

Synergistic interactions could accelerate the evolution of antibiotic resistance by generating stronger selection for resistance, due to increased inhibition of the sensitive strain. Consistent with this idea, we found that synergistic combinations had a greater inhibitory effect on the sensitive ancestral strain (i.e. the wild type strain first exposed to antibiotics at the beginning of the experiment) than antagonistic combinations (Fig. 3.4A; $F_{1, 12} = 37.9$, $P < 0.001$). This was an overall F test. Populations with a lower growth rate of the sensitive ancestral strain showed more adaptation (Fig. 3.4B; $F_{1, 12} = 36.8$, $P < 0.001$). This was an overall F test. Thus, the more synergistic an antibiotic

interaction, the greater the rate of adaptation (Fig. 3.4C; $F_{1, 12} = 10.6$, $P < 0.01$). This was an overall F test.

After controlling for the confounding effect of growth rate of the sensitive ancestral strain, we found that antibiotic interactions do not influence the rate of adaptation ($F_{2,11} = 36.8$ for full model, for specific treatment contrast $t = -3.88$, and $P = 0.33$). Thus the effect of interactions on the singly-resistant mutant is not a significant influence on adaptation in our system.

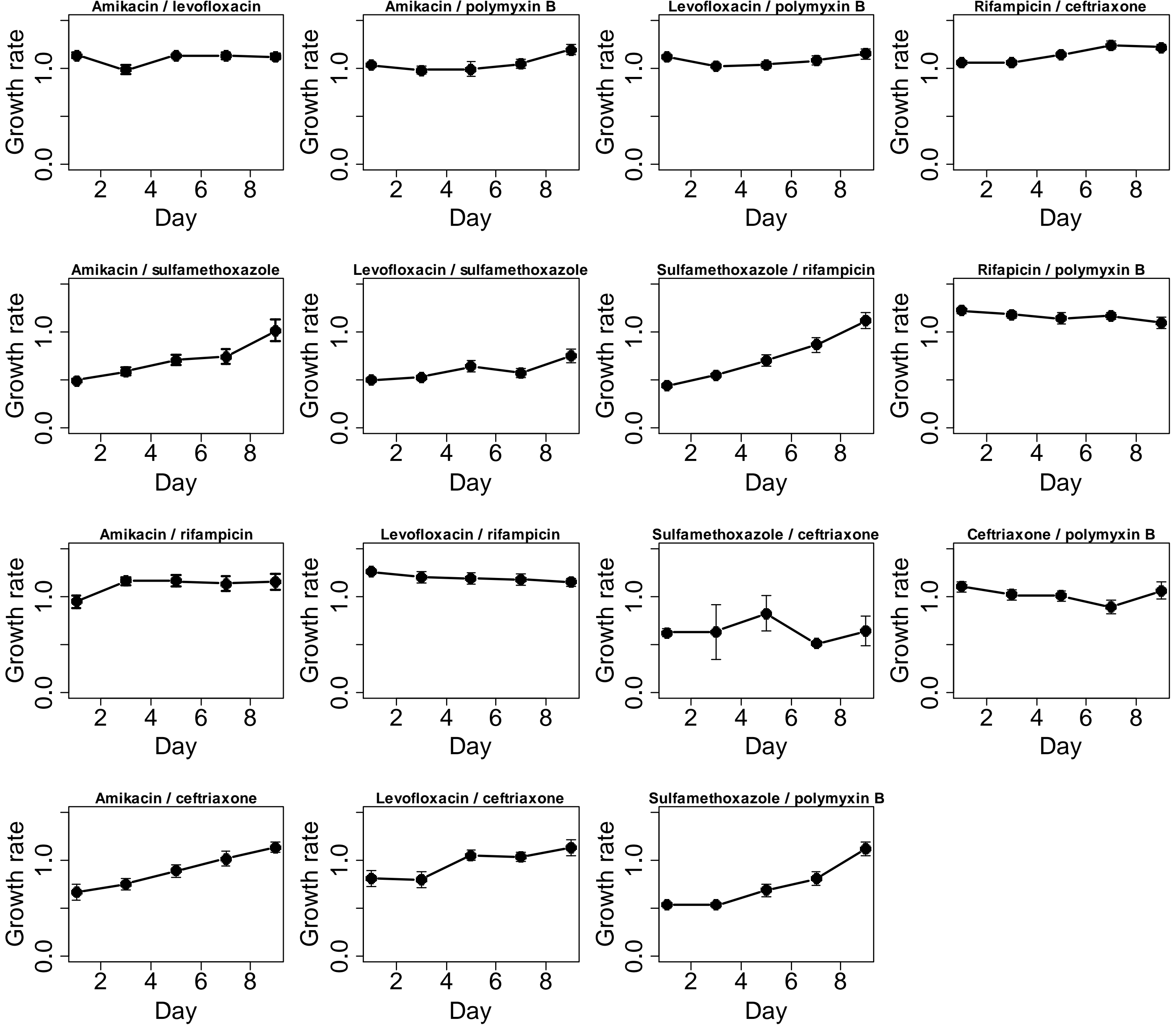


Figure 3.3a. Plots of Day v. Growth rate for populations exposed to each treatment combination. Data points are mean values +/- s.e.m. across populations for each treatment.

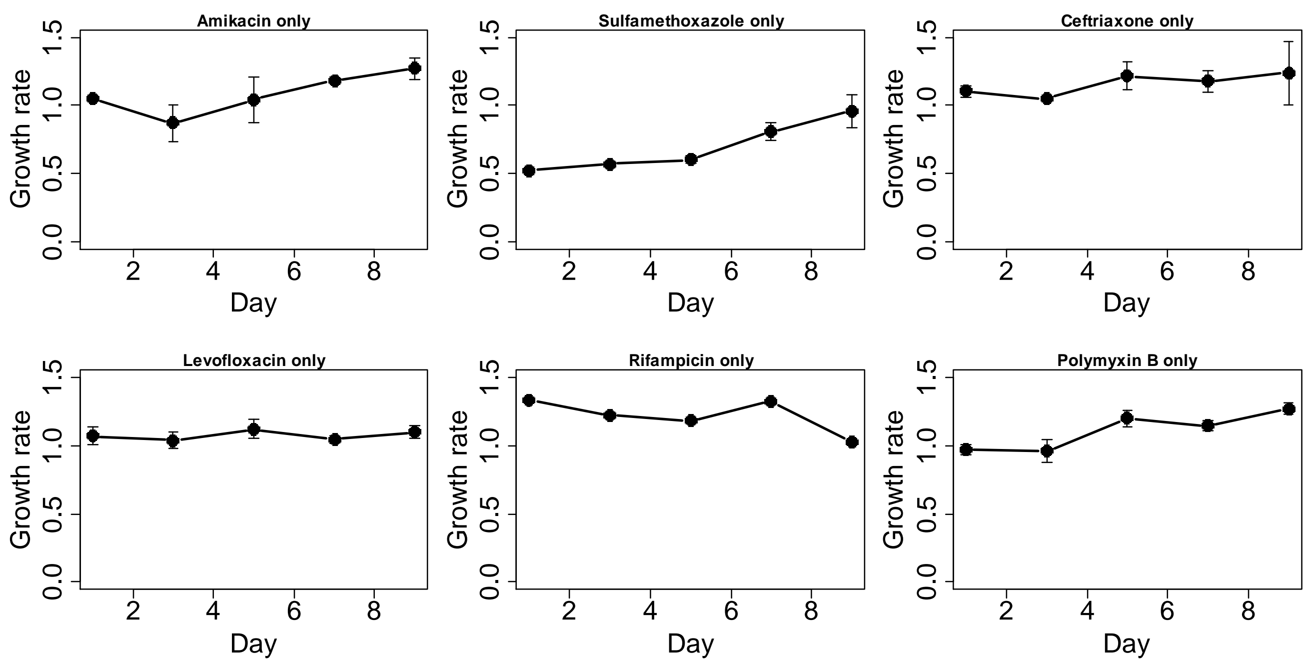


Figure 3.3b. Plots of Day v. Growth rate for populations exposed to each individual antibiotic treatment. Data points are mean values +/- s.e.m. across populations for each treatment.

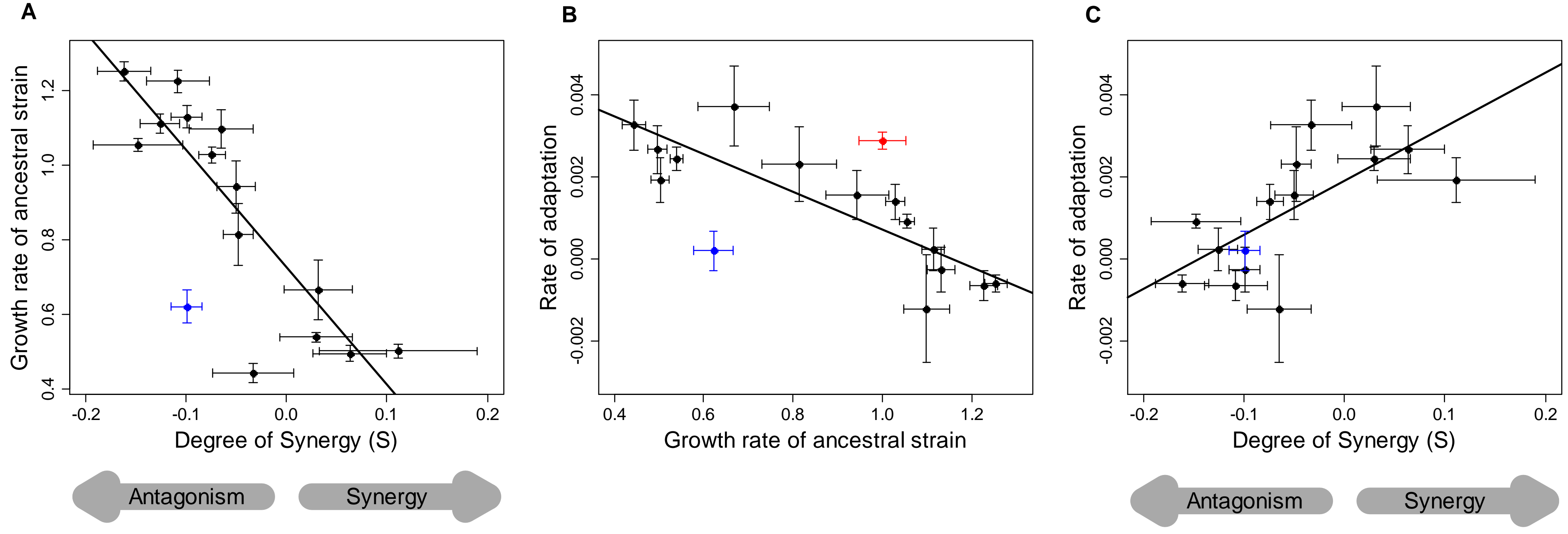


Figure 3.4. Linking antibiotic interactions to the evolution of resistance. Plotted points in (*A*) show the relative growth rate of the sensitive ancestral strain in different antibiotic combinations as a function of degree of synergy. Panel (*B*) shows the rate of adaptation to antibiotic combinations as a function of the growth rate of the sensitive ancestral strain in each combination. Panel (*C*) shows the rate of adaptation to antibiotic combinations as a function of degree of synergy. Rate of adaptation is measured as change in growth rate per hour of active growth. All plotted points show the mean +/- s.e.m. for a single antibiotic combination and the blue symbol denotes a single antibiotic combination (ceftriaxone/sulfamethoxazole) that was excluded from the analysis as only two populations had survived this treatment. The red symbol ((*B*) only) denotes an antibiotic-free control. Solid black lines show best fit linear regressions.

If degree of synergy is important in determining the rate of evolution of antibiotic resistance only because of its effect on the sensitive strain, then the rate of adaptation of bacteria exposed to individual antibiotics should correlate to the growth rate of the sensitive ancestral strain of these populations. Consistent with this idea, we found that rate of adaptation did correlate to growth rate of the sensitive ancestral strain for bacteria exposed to individual antibiotics ($F_{1,13} = 7.28$, $P < 0.05$, this was an overall F test), and we were unable to distinguish statistically between the rate of adaptation to combinations of antibiotics and to individual antibiotics with the same initial inhibitory effect (Fig. 3.5; $F_{2,26} = 16.83$ for the overall F test, for specific treatment contrast $t = 0.83$ and $P = 0.41$).

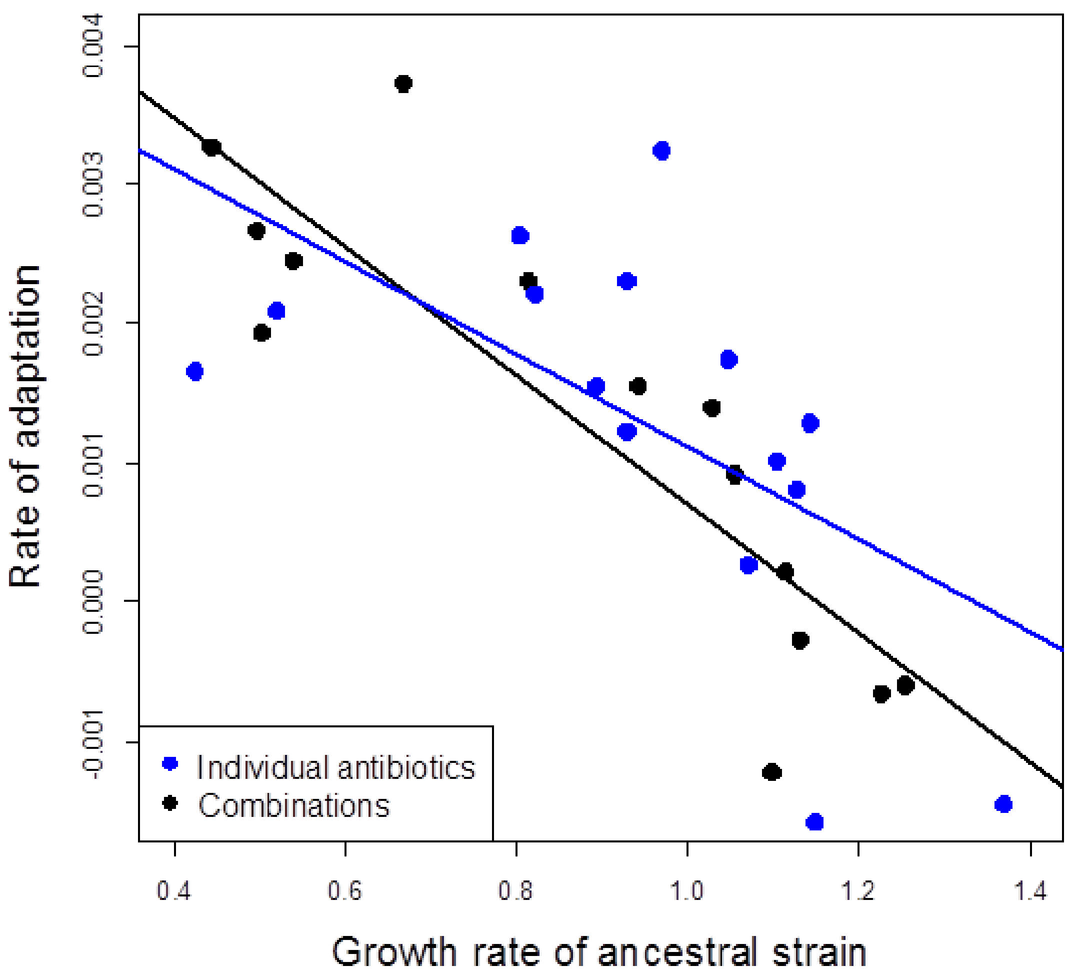


Figure 3.5. More inhibitory antibiotic treatments accelerate resistance evolution. Plotted points show the rate of evolution of antibiotic resistance as a function of initial inhibitory effect (i.e. growth rate of the sensitive ancestral strain) for individual antibiotics (blue dots, n=15) and antibiotic combinations (black dots, n=14). Rate of adaptation is measured as change in growth rate per hour of active growth. Blue and black lines show the linear regression of rate of adaptation against the growth rate of the sensitive ancestral strain, for individual antibiotics and antibiotic combinations respectively.

3.3.3 Testing for cross-resistance

One possible explanation for why degree of synergy does not influence the rate of adaptation to antibiotic combinations through an effect on singly-resistant mutants is that resistance evolves by multiple-antibiotic resistance mutations. To assess the level of cross-resistance present in our system, we took bacteria which had evolved resistance to a single β-lactam, aminoglycoside or quinolone antibiotic, and assayed the growth rate of these bacteria when exposed to an antibiotic from the same range of antibiotic families. Bacteria evolved with β-lactams had a higher growth rate in a β-lactam containing environment than control clones evolved in antibiotic-free conditions (Fig. 3.6A; $t_{14} = 5.10$, $P < 0.001$), indicating that bacteria had acquired resistance during the course of evolution. Likewise, bacteria which had evolved with aminoglycosides had a higher growth rate in an aminoglycoside containing environment than the antibiotic-free controls (Fig. 3.6B; $t_{10} = 3.08$, $P < 0.05$), and bacteria which had evolved with quinolones had a higher growth rate in a quinolone containing environment than the antibiotic-free controls (Fig. 3.6C; $t_{18} = 2.11$, $P < 0.05$). None of the bacteria evolved with an antibiotic from a different family than the assay antibiotic displayed a significantly higher growth rate than the control bacteria when exposed to the assay antibiotic, indicating there was no cross-resistance in our system, at least for the antibiotics tested.

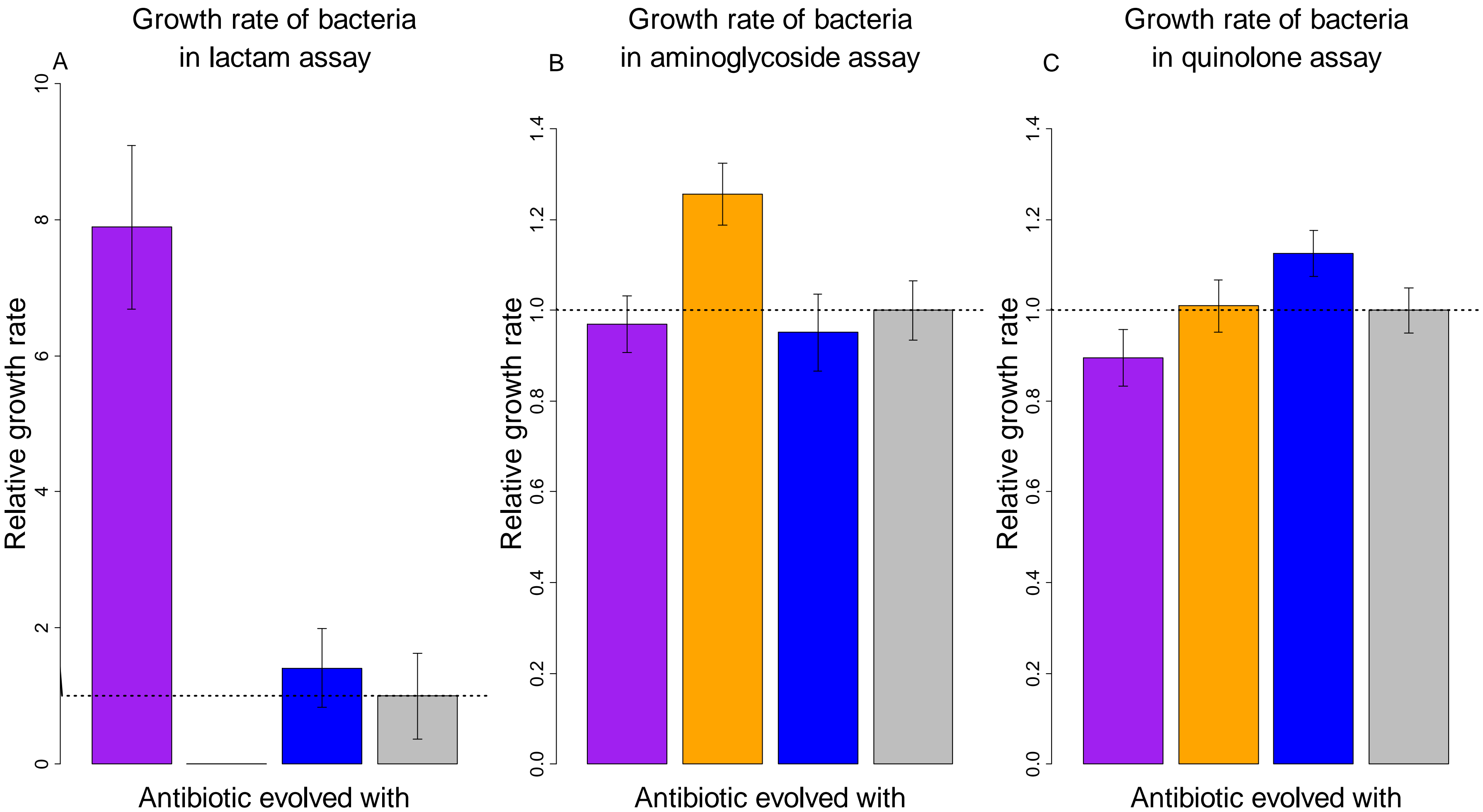


Figure 3.6. Testing for cross-resistance. To test for cross-resistance, we measured the growth rate of evolved bacteria across a range of antibiotics. Bars show the mean (+/- s.e.m.) growth rate of evolved bacteria in the presence of a β-lactam (panel (*A*); ceftazidime), an aminoglycoside (panel (*B*); kanamycin) or a quinolone (panel (*C*); sparfloxacin). Purple bars represent bacteria evolved with a β-lactam antibiotic, yellow bars bacteria evolved with an aminoglycoside antibiotic, blue bars bacteria evolved with a quinolone antibiotic, and grey bars controls evolved in antibiotic-free conditions. Growth rates were standardised such that the mean growth rate of clones that evolved in the absence of antibiotics was set to 1.

3.4 Discussion

In summary, we found that synergistic interactions accelerate the rate of adaptation to antibiotic combinations in the pathogenic bacterium *P. aeruginosa* (see Fig. 3.4C). We obtained this result because synergistic combinations of antibiotics caused more effective inhibition of bacterial growth, leading to stronger selection for resistance (see Fig. 3.4A). After correcting for the increased inhibition of growth associated with synergistic combinations, we did not find any evidence to support the idea that antibiotic interactions impact the rate of evolution of resistance. This important result contradicts influential work arguing that synergy accelerates the evolution of resistance ([68, 113]).

It is a well established principle that a stronger selection pressure will drive a faster rate of adaptation [133] and this has been convincingly demonstrated for antibiotic selection pressures specifically [42, 134]. Our result that combinations which were more inhibitory to the sensitive ancestral strain (i.e. the wild type strain first exposed to antibiotics at the beginning of the experiment) accelerated the evolution of resistance is consistent with this principle; when the sensitive strain has a lower growth rate, the proportional selective advantage of resistance is greater and so resistant mutants will spread more quickly in the population. By definition, synergistic combinations are more inhibitory than antagonistic combinations for a given concentration of antibiotics, and so pose a stronger selective pressure for resistant mutants.

There are a few possible explanations for our finding that the effect of degree of synergy on singly-resistant mutants did not drive evolution in our system. It may be that

the effect of degree of synergy on the singly-resistant mutant has only a very weak effect on the evolution of resistance and we did not have the power to detect the effect in our experiment. This explanation is supported by the fact that Hegreness *et al.* found only a weak correlation in their *Escherichia coli* system between degree of synergy and rate of adaptation once drug pair membership and initial growth rate (the impact of the antibiotic combination on the growth rate of the sensitive wild type) were controlled for ($\rho = 0.249$, indicating that the effect of degree of synergy on the growth rate of singly-resistant mutants explained about 6% of the variation in the rate of adaptation) [68]. Our experiment, however, was carried out on the largest scale attempted so far (6 antibiotics and 15 antibiotic combinations) and so we might have expected to detect an effect if one was present.

Cross-resistance has been shown to be important in determining the influence of antibiotic combinations on adaptation, in addition to degree of synergy. In work by Michel *et al*. [113], *Staphylococcus aureus* acquired a mutation conferring resistance to both antibiotics in an antagonistic combination, but did not acquire a mutation conferring resistance to both antibiotics in an additive or a synergistic combination. The antagonistic combination therefore appeared to drive faster evolution of resistance, until the effects of cross-resistance and degree of synergy were teased apart through a mathematical model. However, we did not find any cross-resistance in our system (see Fig. 3.6). It may be that, while mutations conferring multiple-antibiotic resistance are costly and selected against when bacteria are exposed to just one antibiotic, such mutations are beneficial when bacteria are exposed to combinations of antibiotics. If single mutations conferring multiple-antibiotic resistance are common in our system, the importance of any differential effect of combinations on mutants resistant to one

antibiotic in the combination may be reduced. Alternatively, the degree of synergy experienced by resistant mutants may differ from that experienced by the sensitive wild type; Comber *et al.* found that synergy was most commonly observed with sensitive *P. aeruginosa* strains and was rarely seen with highly resistant strains [135], however other authors have found that synergy is similar for sensitive strains and strains resistant to one antibiotic in a combination [136].

The hypothesis that interactions between antibiotics affect the evolution of antibiotic resistance is of clinical significance. Previous work has argued that synergistic combinations, favoured by clinicians for their increased efficacy for a given concentration, may actually increase the rate of evolution of resistance, whereas overlooked antagonistic combinations may have the potential to delay or prevent the evolution of resistance. Our work suggests, however, that the potential for interactions between antibiotics in combination to offer new treatment strategies has been overstated (see for example [68, 103, 137]). Synergistic combinations are more inhibitory to sensitive wild type bacteria, increasing the selection pressure for resistance, whereas antagonistic combinations impose a lesser selective pressure, but clinicians must achieve an appropriate level of inhibition of bacteria whether through use of a combination or a sufficient concentration of a single antibiotic. In other words, our study argues that there is no added value that comes from using a combination of antibiotics as opposed to a single antibiotic, and this may help to explain why clinical studies report contradictory findings regarding the efficacy of antibiotic combinations ([67, 95-102]).

Chapter 4

4 Effectiveness of a combination of the antibiotics ceftriaxone and sulfamethoxazole in preventing the evolution of antibiotic resistance

4.1 Introduction

Treatments involving antibiotic combinations may be a promising option to combat the ever-growing clinical problem of antibiotic-resistant pathogens [16, 17]. The aims of using antibiotic combinations are to achieve more complete inhibition of bacteria than use of a single antibiotic on its own, maintain effective treatment even when bacteria are resistant to one of the antibiotics in a combination and prevent the evolution of antibiotic resistance [9, 16]. Clinical studies into the effectiveness of antibiotic combinations have not, however, provided conclusive evidence of the benefits of combinations [67, 95-102]. When selecting antibiotics to use together in combination, it is beneficial if the combination of antibiotics is more difficult for bacteria to evolve resistance to than either antibiotic on its own, in order to extend the effective lifespan of antibiotics. It has been proposed that antagonistic combinations of antibiotics may offer this benefit [68], though the evidence is far from conclusive.

In our previous work (see Chapter 3), we tested the rate of evolution of resistance of *Pseudomonas aeruginosa* to various combinations of antibiotics. We found that

resistance to a combination of the antibiotics ceftriaxone and sulfamethoxazole evolved more slowly than would be predicted based on the inhibitory effect of this combination on sensitive ancestral bacteria (i.e. on the growth rate of the wild type strain when it was first exposed to antibiotics) (see Fig. 3.4B). Only two of eighteen populations exposed to this antibiotic combination survived to the end of the selection experiment, so our result was not conclusive (though the extinction of populations indicated the potency of this combination of antibiotics). We decided that further investigation into this combination was warranted.

Ceftriaxone is a β-lactam antibiotic; it binds penicillin-binding proteins and prevents normal cross-linking of peptides in the bacterial cell wall [21]. Sulfamethoxazole is a member of the sulphonamide family of antibiotics and inhibits folate synthesis [130]. B-lactam / sulfamethoxazole combinations are not generally used clinically and so little information is available about the effectiveness of this combination or how it might affect the evolution of antibiotic resistance. It is unclear why this combination of antibiotics would delay the evolution of resistance. It has been proposed that β-lactam antibiotics may increase the permeability of the cell to other antibiotics, thus increasing the intra-cellular concentration of a secondary antibiotic [23]. By keeping the bacterial population size very small, a ceftriaxone / sulfamethoxazole combination may therefore reduce the supply rate of resistance-conferring mutations, and increase the chance of resistance mutations being lost through stochastic effects before they become established in the population [33]. However, our previous work (see Chapter 3) indicates that the two antibiotics have a mildly antagonistic interaction at the concentrations used in that work (i.e. the two antibiotics do not strongly modify each other's inhibitory effects).

To test the effectiveness of a combination of ceftriaxone and sulfamethoxazole in preventing the evolution of antibiotic resistance, we conduct an experimental evolution study using the important opportunistic human pathogen *P. aeruginosa*. We experimentally challenge *P. aeruginosa* with adapting to ceftriaxone and sulfamethoxazole separately, and to combinations of the two antibiotics, and test for differences in the rate of adaptation. As the effectiveness of antibiotic combinations can be dependent on the concentration of each antibiotic relative to the other [119, 121], we create a grid of combinations with each antibiotic at different concentrations. Using this approach, we find that the rate of adaptation to a combination of ceftriaxone and sulfamethoxazole is no different than the rate of adaptation to either antibiotic on its own, once the inhibitory effect of each treatment on the sensitive ancestral strain is controlled for.

4.2 Materials and Methods

4.2.1 Conditions, strain and antibiotics used

All experiments were conducted in Mueller-Hinton 2 liquid media (Fluka 90922), with bacteria grown at 37°C. The populations were founded from a single clone of luminescent-tagged *P. aeruginosa* PA01: mini-Tn7T-Gm-*lux*, as described in Chapter 3.

The two antibiotics used were ceftriaxone (Sigma C5793) and sulfamethoxazole (Sigma S7507); solutions were made up from powder stocks. Ceftriaxone concentrations used were: 5 mg/l for the low concentration, 7.5 mg/l for the medium concentration, and 10

mg/l for the high concentration. Sulfamethoxazole concentrations used were: 50 mg/l for the low concentration, 125 mg/l for the medium concentration, and 200 mg/l for the high concentration. A grid of antibiotic treatments was created as in Fig. 4.1 below. 22 replicate populations were exposed to each treatment. The antibiotic concentrations were selected with the intention that the low concentrations of antibiotics would reduce log sensitive bacterial growth rate by approximately 10%, the medium concentrations by approximately 25% and the high concentrations by approximately 50%. Antibiotic concentrations were selected based on previous work and pilot work for this experiment.

		Sulfamethoxazole concentration			
		None	Low	Medium	High
Ceftriaxone concentration	None				
	Low				
	Medium				
	High				

Figure 4.1. Antibiotic treatments included in the selection experiment. Treatments included each antibiotic individually at each concentration, a combination of the antibiotics at each concentration of the two antibiotics, and an antibiotic-free control.

4.2.2 Selection experiment

The selection experiment was set up as described in Chapter 3. Measurements were taken by culturing populations in static conditions and assaying luminescence expression every 20 minutes for 24 hours using a spectrometer (FLUOStar OPTIMA, BMG Labtech). In addition to the luminescence measurements, measurements were

taken using BacTiter-Glo reagent (Promega) on Day 1 and Day 2 and then every two days, taking a sample for measurement just before transfer to fresh media (see Chapter 3 for further details of the BacTiter-Glo reagent). The experiment was continued for eight days.

The plates used were black 384-well plates (black plates were used in order to minimise cross-talk between wells). The culture volume in each well was 80µl. Please see Appendix B for the layout of the treatments within each 384 well plate- this layout was chosen to reduce the time taken to setup the experimental plate. The FLUOStar OPTIMA spectrometer read plates in the pattern indicated in Appendix B. The Precision XS automated pipettor multi-channel function was used to transfer 8 cultures at a time during plate transfers, as indicated in Appendix B.

4.2.3 Determination of growth rate of sensitive ancestral strain and rate of adaptation

In this thesis, we refer to the $\log_{10}$ final density measurements produced using Bac-Titer Glo as 'growth rate' measurements, as they are a proxy of the number of cell doublings a population has undergone during a 24 hour period (see Appendix A for a validation of the use of Bac-Titer Glo measurements). The growth rate of the sensitive ancestral strain exposed to each treatment (i.e. the inhibitory effect of each treatment on sensitive wild type bacteria when first exposed to the treatment) is likely to have affected the rate of adaptation, as discussed in Chapter 3. To control for this, we calculated the growth rate of the sensitive ancestral strain and included it as a term in our statistical analyses.

Growth rate of the sensitive ancestral strain was calculated as $f_{day1} = f_{treatment\ Day1} / f_{control\ Day1}$, where $f_{treatment\ Day1}$ is growth rate of bacteria exposed to a given antibiotic treatment on Day 1 of the selection experiment, and $f_{control\ Day1}$ is growth rate of a control grown in antibiotic-free conditions on Day 1.

To calculate the rate of adaptation, we followed the method proposed by Hegreness *et al*. [68] as described in Chapter 3. Growth rate values used were log_{10} BacTiter-Glo (Promega) measurements as described above. Half the total adaptation was calculated as $(0.5\ ((f_{treatment\ Day8} / f_{control\ Day1}) - (f_{treatment\ Day1} / f_{control\ Day1})))$.

4.2.4 Statistical analyses

All statistical analyses were carried out using R Version 2.13.2 [131]. First, populations which did not show Day 8 BacTiter-Glo (Promega) values above those of control wells containing media only were excluded; this was a high proportion of populations for the higher concentration antibiotic treatments (Fig. 4.2).

We used the population survival rate as a measure of the effectiveness of each treatment. We calculated survival rate as the number of populations which showed Day 8 BacTiter-Glo (Promega) values above those of control wells containing media only, divided by the number of replicate populations at the start of the experiment (22 for each treatment). We fitted a model testing whether survival rate was determined by the concentration of each antibiotic. We then fitted a model to test how growth rate of the sensitive ancestral strain, and a term coding for whether a treatment was an individual

antibiotic or a combination treatment, and the interaction between these two terms, affected the survival rate. As the interaction term was not significant, we removed it to fit the minimum adequate model.

To test how the antibiotic concentration affected the growth rate of the sensitive ancestral strain, we used a general linear model with the concentration of ceftriaxone and the concentration of sulfamethoxazole as terms in the model. We then fitted another model to test how growth rate of the sensitive ancestral strain, and a term coding for whether a treatment was an individual antibiotic or a combination treatment, and the interaction between these two terms, affected the rate of adaptation. As the interaction term was not significant, we removed it to fit the minimum adequate model.

We carried out a linear regression to test for a correlation between rate of adaptation and survival rate. Finally, some antibiotics may be more difficult for bacteria to evolve resistance to than others [138]. We tested for such an effect in our system by fitting models comparing the rate of adaptation of bacteria exposed to each antibiotic individually, and the survival rate of bacteria exposed to each antibiotic individually, controlling for the growth rate of the sensitive ancestral strain. The growth rate of the sensitive ancestral strain was controlled for by including it as a term in the model.

4.3 Results

To test the hypothesis that a combination of the antibiotics ceftriaxone and sulfamethoxazole was more difficult for bacteria to evolve resistance to than would be

predicted by the rate of adaptation to each antibiotic individually, we carried out a selection experiment where we exposed bacterial populations to ceftriaxone and sulfamethoxazole individually and in combination, at a number of different antibiotic concentrations (Fig. 4.2). Higher concentrations of antibiotics, whether in combination or individually, resulted in lower population survival rates ($F_{2,12} = 24.13$ for overall F test. For specific treatment contrasts, $t = -5.64$, $P < 0.001$ for ceftriaxone, and $t = -4.83$, $P < 0.001$ for sulfamethoxazole). For five combinations where populations were exposed to higher antibiotic concentrations, no populations survived to Day 8 of the selection experiment. The lower the growth rate of the sensitive ancestral strain (i.e. the lower the growth rate of the wild type strain when it was first exposed to antibiotics on Day 1 of the experiment), the lower the survival rate (Fig. 4.3B; $F_{1,8} = 12.25$, $P < 0.01$). This was an overall F test. Once the effect of growth rate of the sensitive ancestral strain on survival rate was controlled for, the difference between the survival rate in individual antibiotic and in combination treatments did not reach significance ($F_{2,7} = 10.91$ for overall F test, for specific treatment contrast $t = 2.10$ and $P = 0.07$). There was also no significant interaction between growth rate of the sensitive ancestral strain and whether a treatment included an individual antibiotic or an antibiotic combination ($F_{3,6} = 6.81$ for overall F test, for specific treatment contrast $t = -0.65$ and $P = 0.54$).

Higher concentrations of antibiotics, whether applied individually or in combination, resulted in lower growth rate of the sensitive ancestral strain ($F_{2,7} = 6.32$ for the overall F test, for the specific treatment contrasts, $t = -2.77$, $P < 0.05$ for ceftriaxone, $t_7 = -3.38$, $P < 0.05$ for sulfamethoxazole). Populations with a lower growth rate of the sensitive ancestral strain experienced a greater selection pressure for resistance and adapted more rapidly (Figs. 4.2 and 4.3A; $F_{1,8} = 15.25$, $P < 0.01$). This was an overall F test. After

controlling for the growth rate of the sensitive ancestral strain, we could not distinguish between the rate of adaptation to individual antibiotics and the rate of adaptation to antibiotic combinations ($F_{2,7} = 6.71$ for overall F test, for specific treatment contrast t = 0.15 and P = 0.88). Additionally, we did not find an interaction between the growth rate of the sensitive ancestral strain and whether a treatment was an individual antibiotic or combination treatment ($F_{3,6} = 4.29$ for overall F test, for specific treatment contrast t = 0.69 and P = 0.52). We therefore conclude that the combination of ceftriaxone and sulfamethoxazole is not better at preventing the evolution of antibiotic resistance than predicted by the rate of adaptation of bacteria to each antibiotic individually.

We found a negative correlation between rate of adaptation and survival rate, so that in treatments where survival rate was lower, surviving populations showed more adaptation (Fig. 4.3C; $F_{1,9} = 6.96$, $P < 0.05$). This was an overall F test. Once growth rate of the sensitive ancestral strain had been controlled for, we did not find a difference in the rate of adaptation to ceftriaxone and to sulfamethoxazole in the populations exposed to one antibiotic only ($F_{2,3} = 3.76$ for overall F test, for specific treatment contrast t = 0.94 and P = 0.41), or in the survival rate ($F_{2,3} = 4.53$ for overall F test, for specific treatment contrast t = 0.63 and P = 0.58); however our sample size was small (see Materials and Methods).

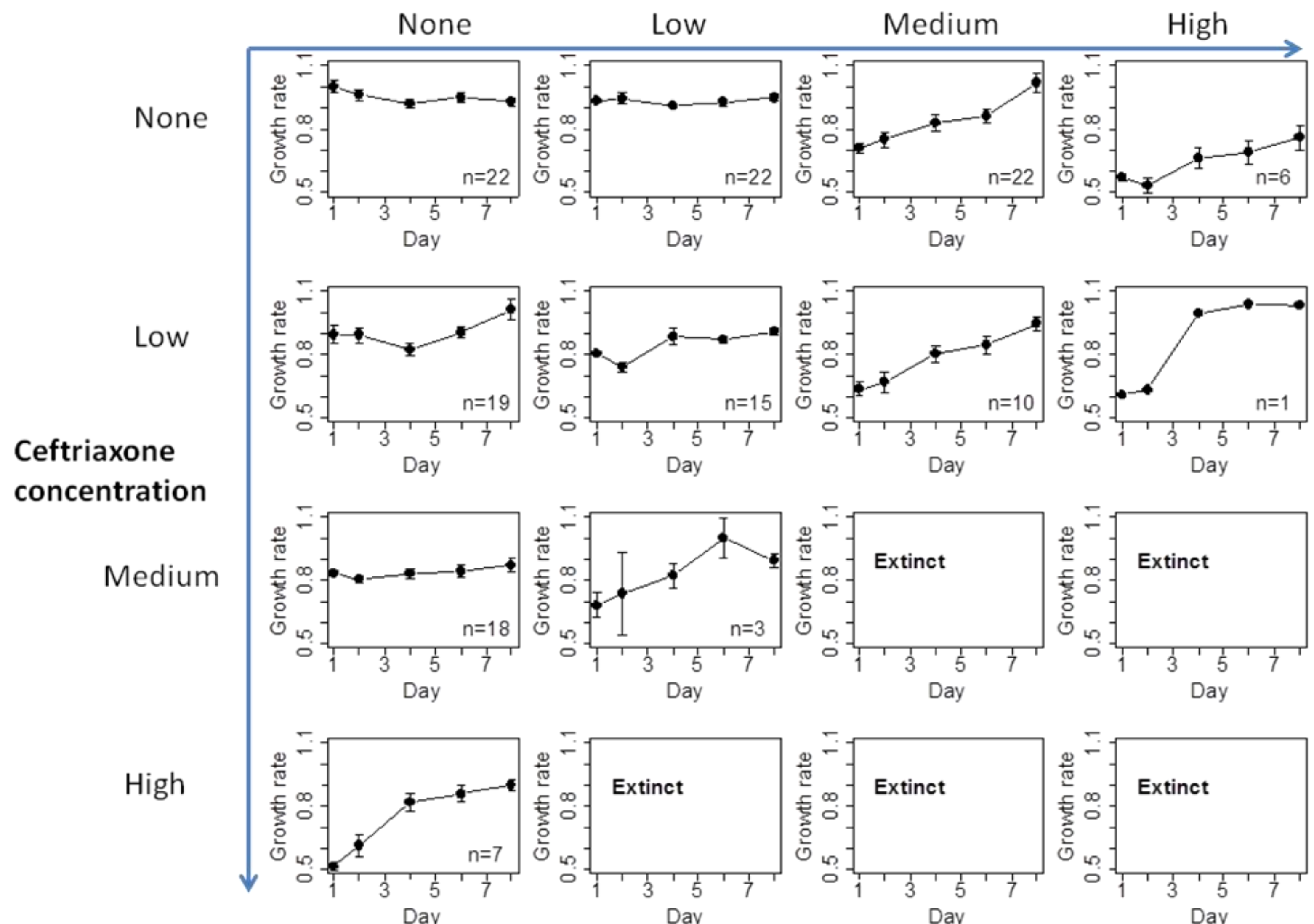


Figure 4.2. Plots of Day v. Growth rate for populations exposed to each treatment. Data points are mean values +/- s.e.m. across populations for each treatment. n values are the number of populations surviving to Day 8 for each treatment (out of 22 replicate populations starting in each treatment). In five treatments marked as 'Extinct', no populations survived to the end of the selection experiment.

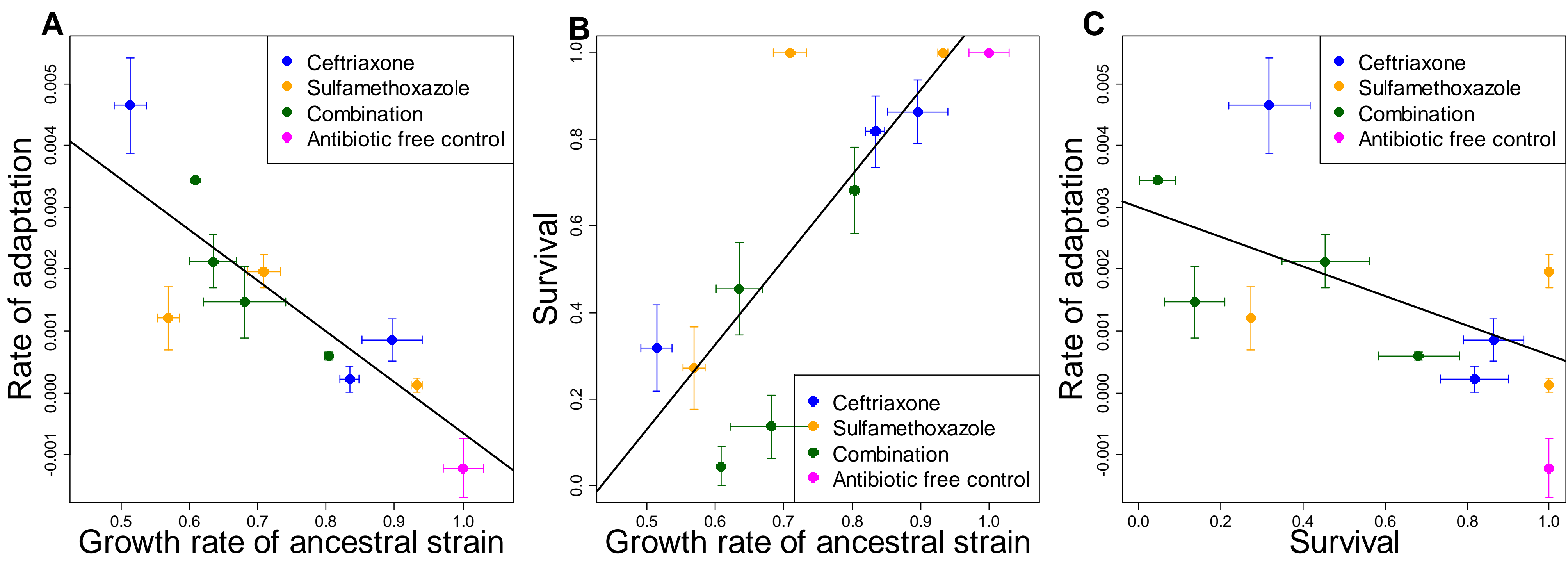


Figure 4.3. Correlations between rate of adaptation, survival and growth rate of sensitive ancestral strain. Panel *(A)* shows the rate of adaptation to antibiotic treatments as a function of the growth rate of the sensitive ancestral strain in each treatment. Panel *(B)* shows the population survival rate in each antibiotic treatment as a function of the growth rate of the sensitive ancestral strain in each treatment. Panel *(C)* shows the rate of adaptation to antibiotic treatments as a function of the population survival rate in each antibiotic treatment. Rate of adaptation is measured as change in growth rate per hour of active growth. Data points are mean values +/- s.e.m. across populations for each treatment.

4.4 Discussion

In summary, we found that a combination of the antibiotics ceftriaxone and sulfamethoxazole was no better at preventing the evolution of antibiotic resistance than either antibiotic used on its own, for a given growth rate of the sensitive ancestral strain (i.e. for a given initial inhibitory effect of an antibiotic treatment). This result held at all concentrations of the two antibiotics applied (see Fig. 4.2). Our hypothesis that this combination would be more effective than expected at preventing the evolution of resistance was based on a small number of samples from our previous work in Chapter 3. Our work here shows that our hypothesis was not correct.

Consistent with established principle (see for example [42, 133, 134]) and our work in Chapter 3, we find that the inhibitory effect of a treatment on the sensitive ancestral strain is strongly correlated with the rate of adaptation. When fitness of the sensitive ancestral strain is lower, there is a greater selective advantage to resistance, and so resistant strains will spread more rapidly in the population. An interesting feature of our results is the negative correlation between rate of adaptation and survival rate. When populations were less likely to survive an antibiotic treatment, as in the case of the high-concentration combination treatments, those populations which did survive showed more adaptation. Again, this is consistent with previous work which shows that a very strong selection pressure drives faster adaptation in those populations which escape extinction [139].

Although we did not find that the combination of ceftriaxone and sulfamethoxazole was more effective than expected in delaying the evolution of resistance, we did find the combination to be tractable and to allow accurate control of growth rate of the sensitive ancestral strain by altering antibiotic concentration, and so we decided to use this combination for our final project on antibiotic heterogeneity (see Chapter 5).

Chapter 5

5 Higher levels of antibiotic heterogeneity accelerate the evolution of antibiotic resistance, in a *Pseudomonas aeruginosa* experimental system

5.1 Introduction

Manipulating environmental heterogeneity lies at the heart of evolution-based strategies to combat the rise of antibiotic resistance in bacteria [9, 15]. Antibiotic heterogeneity may reduce the selection pressure for resistance to any one antibiotic and thus reduce the spread of resistant organisms. It is also hoped that antibiotic resistant strains may reduce in frequency when they compete with antibiotic sensitive strains in the absence of a particular antibiotic. A classic question in the antibiotic literature is whether the antibiotics used in a hospital should be 'cycled', or 'mixed'. In antibiotic 'cycling' all patients in a ward are given the same antibiotic, and the antibiotic used is rotated periodically, eventually returning to the first antibiotic, so that pathogens encounter different antibiotics over time. Antibiotic 'mixing' is where different patients in a hospital are assigned different antibiotics, so that pathogens colonising a new host may find the antibiotic treatment is different to the one they had previously encountered.

Mathematical models have predicted that the more heterogeneous an environment, the less evolution of resistance will occur [73, 140]. In the classical model of cycling and mixing, mixing is more effective in minimising the rate of evolution of resistance [73, 140], which Bergstrom *et al.* [73] explain by suggesting that bacterial clones will encounter more heterogeneity in a mixed environment than a cycling program where cycles may last many months. These model results were supported by a clinical study which found that high heterogeneity treatments (mixing rather than cycling) resulted in a lower incidence of antibiotic resistance in various pathogens [141]. However, another clinical study found that a lower level of heterogeneity (cycling rather than mixing) reduced the rate at which patients acquired β-lactam resistant *Pseudomonas aeruginosa* [142].

A number of clinical trials have been carried out comparing antibiotic cycling to a 'business-as-usual' scenario; the majority of studies have not found cycling to be effective in reducing the proportion of bacterial isolates which show antibiotic resistance [15]. One study linked antibiotic cycling to an outbreak of multiple-antibiotic resistant *P. aeruginosa* [143]. It may be that heterogeneity is more beneficial in preventing the evolution of resistance when the switch to a new antibiotic is made based on the level of resistance reached in a clinical setting ('adjustable cycling'), rather than after a fixed time interval [144-146]. Confounding factors such as the introduction of new infection control methods during study periods and prioritisation of other considerations over following an experimental regime [140], in addition to the potential for different effects depending on the pathogen species involved [138, 145], make it difficult to draw conclusions about how antibiotic heterogeneity may affect the evolution of resistance in clinical situations. Overall, evidence demonstrating the

benefit of antibiotic heterogeneity in preventing the evolution of resistance is not conclusive [15, 69, 70].

A study into the adaptation of the single celled alga, *Chlamydomonas reinhardtii,* when exposed to constant light, constant dark, or fluctuating light and dark environments, produced some interesting results [147]. Populations evolved in fluctuating environments grew almost as well in the light as populations evolved in the constant light environment, and also showed adaptation to the dark environment. Populations evolved at two different rates of environmental fluctuation showed very similar fitness gains. These results would suggest that increasing environmental heterogeneity does not reduce the rate of adaptation.

Despite the importance of understanding how heterogeneity in the antibiotic environment affects the evolution of resistance, relatively few experimental studies have been conducted. Here, we carry out a selection experiment in *P. aeruginosa* where we expose bacteria to environments with varying degrees of heterogeneity. We create environments where the antibiotic present is rotated every day, two days or four days (see Fig. 5.1), with shorter cycle times creating a higher degree of heterogeneity. While previous experimental evolution studies have compared the evolution of resistance in a heterogeneous antibiotic environment to the evolution of resistance in a constant antibiotic environment [148, 149], this is, to our knowledge, the first time a laboratory-based study has been carried out testing how different rates of fluctuation within a heterogeneous environment affect the rate of evolution of antibiotic resistance.

5.2 Materials and Methods

5.2.1 Conditions, strain and antibiotics used

All experiments were conducted in Mueller-Hinton 2 liquid media (Fluka 90922), with bacteria grown at 37°C. The populations were founded from a single clone of luminescent-tagged *P. aeruginosa* PA01: mini-Tn7T-Gm-*lux*. This strain carries a bioluminescent reporter construct (the *lux* construct) that allows rapid and sensitive measurements of bacterial growth rate across a broad dynamic range (see [108]). *P. aeruginosa* is a model organism in experimental evolution, and an important opportunistic human pathogen that is difficult to treat clinically due to its antibiotic resistance profile [93].

Antibiotics were made up from powder stocks. The two antibiotics used were ceftriaxone (Sigma C5793) and sulfamethoxazole (Sigma S7507). Ceftriaxone is a third generation cephalosporin and a member of the β-lactam family of antibiotics. It binds penicillin-binding proteins and prevents normal cross-linking of peptides in the bacterial cell wall [21]. Sulfamethoxazole is a member of the sulphonamide family of antibiotics, and works by inhibiting folate synthesis [130]. Bacteria were exposed to ceftriaxone at a concentration of 5.9 mg/l and sulfamethoxazole at a concentration of 109 mg/l. The antibiotic concentrations were selected with the intention of reducing log bacterial growth rate by approximately 40%. Antibiotic concentrations were selected based on previous work and pilot work for this experiment.

5.2.2 Selection experiment

To form the populations to be exposed to each treatment, an overnight saturated culture of PA01: mini-Tn7T-Gm-*lux* was diluted into wells of a 384 well plate such that each well contained approximately 100 founding cells. These small founding populations allowed for unique mutants in each population. As the antibiotic concentrations used would be lethal to populations of this size, populations were then allowed to grow to saturation in antibiotic-free media before being diluted to allow approximately 10^5 cells per well, into media containing ceftriaxone, sulfamethoxazole or no antibiotic depending on the treatment each population was assigned to. Measurements were taken by culturing populations in static conditions and assaying luminescence expression every 20 minutes using a spectrometer (FLUOStar OPTIMA, BMG Labtech). Every 24 hours populations were diluted so that approximately 10^5 cells were transferred to the corresponding treatment on a fresh plate with fresh media. The experiment was continued for 16 days. All plate set-ups and transfers were carried out using an automated pipettor (Precision XS, BioTek).

The treatments used were: constant exposure to ceftriaxone, constant exposure to sulfamethoxazole, switching between a ceftriaxone and a sulfamethoxazole environment every 24 hours, switching between a ceftriaxone and a sulfamethoxazole environment every two days, switching between a ceftriaxone and a sulfamethoxazole environment every four days, and a constant antibiotic-free environment as a control. There were 32 replicate populations for each constant antibiotic treatment, 96 replicate populations for each fluctuating treatment, and 16 replicate populations for the antibiotic-free control. In each fluctuating treatment, 48 populations started the

experiment in a ceftriaxone environment and 48 in a sulfamethoxazole environment (see Fig. 5.1).

The plates used were black 384-well plates (black plates were used in order to minimise cross-talk between wells). The culture volume in each well was 80µl. Please see Appendix B for the layout of the treatments within each 384 well plate- this layout was chosen to reduce the time taken to set up the experimental plate. The FLUOStar OPTIMA spectrometer read plates in the pattern indicated in Appendix B. The Precision XS automated pipettor multi-channel function was used to transfer 8 cultures at a time during plate transfers, as indicated in Appendix B.

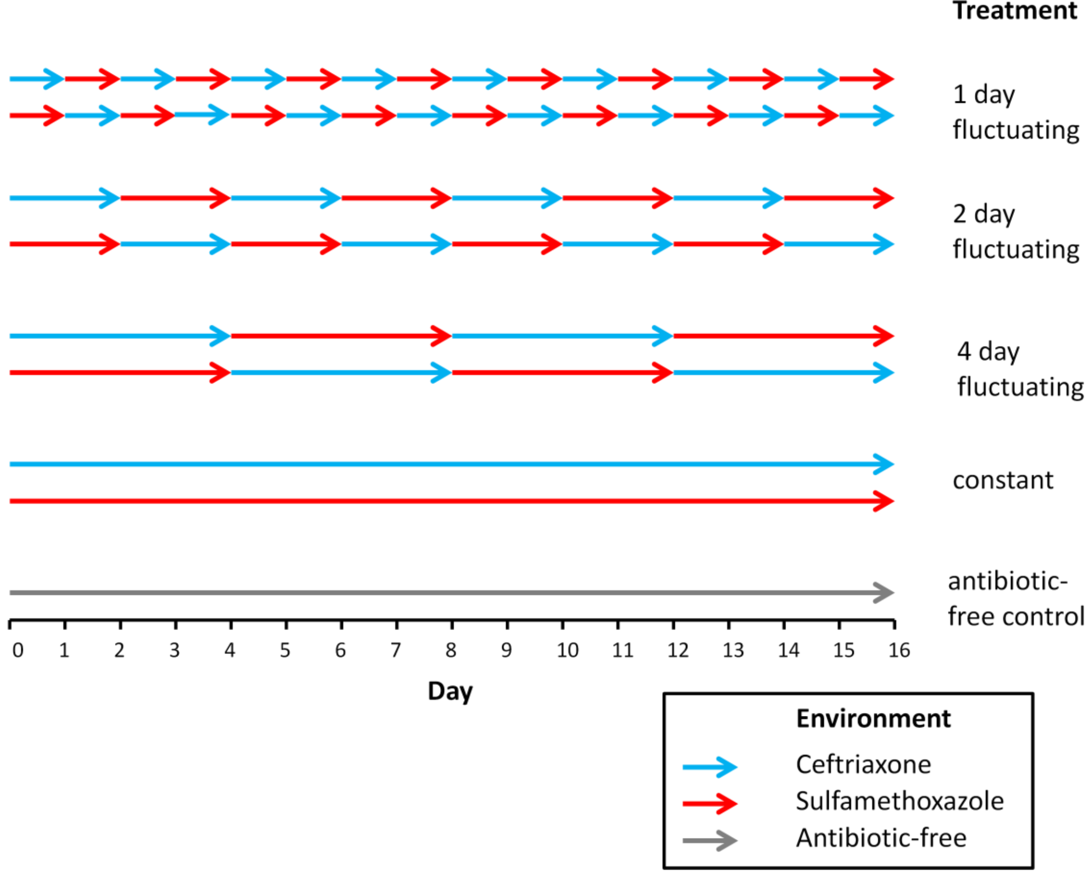


Figure 5.1. Schematic showing the treatments bacteria were exposed to. Treatments were: an environment in which the antibiotic changed every 24 hours, an environment in which the antibiotic changed every 2 days, an environment in which the antibiotic changed every 4 days, a constant ceftriaxone environment, a constant sulfamethoxazole environment, and an antibiotic-free control environment. For each rotating treatment, half of the populations were exposed to ceftriaxone first and half to sulfamethoxazole first.

5.2.3 Determining the rate of adaptation

Exponential growth rates were used as the growth rate values for determination of the rate of adaptation. To calculate exponential growth rates, $\log_{10}$ luminescence - time curves were obtained for each population on each day of the experiment using a spectrometer (FLUOStar OPTIMA, BMG Labtech), and the exponential growth rate calculated as the steepest gradient over a five hour window.

We calculated the rate of adaptation to each antibiotic separately, following the method proposed by Hegreness *et al*. [68]. First we calculated half of the total adaptation a population achieved over the course of the selection experiment. This was calculated as (0.5 (($f_{T\ last\ day}$ / $f_{C\ first\ day}$) - ($f_{T\ first\ day}$ / $f_{C\ first\ day}$))), where $f_{T\ first\ day}$ is the growth rate of a population on the first day of exposure to a particular antibiotic, $f_{C\ first\ day}$ is the growth rate of a control population growing in antibiotic-free conditions on the first day of the selection experiment, and $f_{T\ last\ day}$ is the growth rate of a population on the last day of exposure to a particular antibiotic. We then worked out how long the population took to achieve half the total adaptation. We counted only the time during which a population was actively growing (i.e. in lag or log phases). To calculate the time during each 24 hour period that a population was actively growing, we took the time between the start of luminescence readings on each day and the time at which peak luminescence was reached on that day. We then plotted growth rate on each day against the cumulative time a population had spent actively growing at that point, using R Version 2.13.2 [131] (see Fig. S2 in Appendix A). Interpolation between the data points was carried out using the *approx* function in order to find the cumulative time a population had been actively growing for when it achieved half the total adaptation. The rate of adaptation was then calculated as half the total adaptation divided by the cumulative time taken to achieve half the total adaptation.

Note that the cumulative time a population had spent actively growing was the total cumulative time in any antibiotic environment. An alternative approach would have been to use the cumulative time a population had spent growing in the focal antibiotic only. I decided the total cumulative time was the most appropriate approach as the rate of adaptation to fluctuating treatments was then more in line with the rate of adaptation

to a constant antibiotic treatment, rather than being approximately twice as rapid (see Figure 5.2). Had I used the cumulative time a population had spent growing in the focal antibiotic only, the adaptation rates of populations exposed to treatments with different levels of heterogeneity would still have been similar in relation to each other. However, all populations exposed to heterogeneous treatments would have adapted much more rapidly than populations exposed to a single antibiotic treatment only. I think these results suggest that adaptation to environmental conditions other than the antibiotic was important, most likely adaptation to a new medium- Mueller Hinton 2 broth.

5.2.4 Assays of clones from Day 16 of the selection experiment

To gain further insight into how resistance had evolved, we carried out assays on samples frozen down on the last day of the selection experiment, and on ancestral bacteria. To do this, samples of each population to be assayed were streaked out onto agar plates from freezer stocks. After 24 hours of growth, seven colonies per population were sampled and suspended in antibiotic-free liquid media, to capture seven clones per population (or six colonies to capture six clones per population for the antibiotic-free control). Clones were allowed to replicate for 24 hours. Approximately 10^5 cells per clone were then introduced into the assay environment; each clone was assayed in a ceftriaxone-containing environment, a sulfamethoxazole-containing environment and an antibiotic-free environment. Measurements were taken by culturing populations in static conditions and assaying luminescence expression every 20 minutes for 24 hours using a spectrometer (FLUOStar OPTIMA, BMG Labtech).

On each run of the assay one population evolved with each treatment with each order of antibiotic application was assayed. The assay was run five times to sample five populations per treatment. The level of resistance of each clone was calculated as the growth rate of the clone in a particular antibiotic environment / the growth rate of the clone in an antibiotic-free environment.

5.2.5 Statistical analyses

All statistical analyses were carried out using R Version 2.13.2 [131]. First, populations which did not appear to have grown on Day 16 of the selection experiment were excluded as extinct (roughly 2% of populations). Adaptation to each antibiotic was treated separately in the statistical analyses.

We tested first for differences in the rate of adaptation between the different heterogeneous treatments using a general linear model with each treatment as a continuous variable. The initial growth rate on Day 1 of the experiment of populations exposed to each treatment is likely to have affected the rate of adaptation, as where antibiotics were more inhibitory to the sensitive wild type strain, there would be a stronger selection pressure for resistance and we would expect the evolution of resistance to be more rapid [42, 134]. To control for this, we took $f_{\text{T first day}} / f_{\text{C first day}}$ as the growth rate of the sensitive ancestral strain for each population in each antibiotic and included the growth rate of the sensitive ancestral strain as a term in the model. The model confirmed the influence of the growth rate of the sensitive ancestral strain in each antibiotic on adaptation (populations which had a lower growth rate of the sensitive ancestral strain in ceftriaxone showed more adaptation to ceftriaxone ($F_{3,278}$ =

56.2 for overall F test, for specific treatment contrast $t = -10.06$ and $P < 0.001$), and likewise populations which had a lower growth rate of the sensitive ancestral strain in sulfamethoxazole showed more adaptation to sulfamethoxazole ($F_{3,277} = 90.7$ for overall F test, for specific treatment contrast $t = -9.24$ and $P < 0.001$)). To account for order effects, we also included a term in the model coding for the antibiotic bacteria were exposed to first. However, this term was not significant and could be removed (once the effect of the growth rate of the sensitive ancestral strain in each antibiotic on adaptation had been controlled for, the order in which antibiotics were applied did not affect the rate of evolution of resistance (For adaptation to ceftriaxone: $F_{4,277} = 42.8$ for overall F test, for specific treatment contrast $t = -1.37$ and $P = 0.17$. For adaptation to sulfamethoxazole: $F_{4,277} = 67.8$ for overall F test, for specific treatment contrast $t = 0.131$ and $P = 0.90$)).

We also tested whether bacteria exposed to heterogeneous treatments adapted at a different rate than bacteria exposed to constant treatments, using a general linear model with each treatment as a categorical variable. We included terms in the model coding for the growth rate of the sensitive ancestral strain and the antibiotic bacteria had been exposed to first.

We tested for a correlation between rate of adaptation to ceftriaxone and rate of adaptation to sulfamethoxazole by fitting a general linear model including a term for the treatment used and terms for the growth rate of the sensitive ancestral strain in each antibiotic. To test whether bacteria had adapted more quickly to one antibiotic than the

other, we carried out a t-test on the rates of adaptation of populations exposed to each constant treatment.

To analyse the data from the assays of clones from Day 16 of the selection experiment, we first calculated the mean level of resistance for each population in each antibiotic by averaging across the clones for a particular population. To compare the resistance of populations evolved with each treatment to the resistance of the sensitive ancestral strain, we carried out one-tailed t-tests comparing each treatment to the ancestral strain. To test for cross-resistance in our system, we focused on the populations evolved in constant antibiotic treatments. We ran one-tailed t-tests to check if the resistance of populations evolved with one antibiotic was higher in the alternative antibiotic environment than that of control populations evolved in antibiotic-free conditions. I used t-tests as I wanted to compare the mean resistance of populations evolved with one antibiotic to the mean resistance of control populations evolved in antibiotic-free conditions, when exposed to an antibiotic environment which none of the populations had encountered before. I used one-tailed t-tests as I was only interested in the possibility that the populations evolved with one antibiotic had a higher resistance than the control populations, and not the possibility that the populations evolved with one antibiotic had a lower resistance than the control populations.

Antibiotic resistance will often carry a cost by reducing the growth rate of resistant bacteria in an antibiotic-free environment compared to the growth rate of sensitive bacteria in that environment. We tested for a cost of resistance in the freezer stocks taken from the last day of the selection experiment, by taking the mean raw growth rate

for each population when assayed in antibiotic-free conditions, and using a t-test to check if the mean growth rate for each antibiotic treatment was significantly different to the mean growth rate of a control strain evolved in antibiotic-free conditions.

Note: A standard way to test for the fitness cost of antibiotic resistance is to compare the growth rate of bacteria which are antibiotic resistant to the growth rate of bacteria which are antibiotic sensitive, when each population is grown separately in antibiotic free conditions. To ensure a fair test, I used antibiotic sensitive bacteria which were evolved in conditions identical to the environment the antibiotic resistant bacteria evolved in, apart from the environment of the antibiotic sensitive bacteria did not contain antibiotics. This avoided compounding factors such as bacterial adaptation to the media preventing a direct comparison between the growth rate of the antibiotic sensitive and the antibiotic resistant strains. Comparing the populations evolved with antibiotics to their ancestors would not have provided as robust a test. However, had I compared the populations evolved with antibiotics to the ancestral populations, I would expect to find no cost of resistance (the same qualitative result as comparing to control populations evolved without antibiotics), as the ancestral strain had a lower growth rate than any of the evolved strains (see p109).

To test the hypothesis that higher levels of environmental heterogeneity maintain more genetic variation in populations, we calculated the variance in the level of resistance to each antibiotic across the clones in each population. Fitting each treatment as a continuous variable, we used a general linear model to test how treatment affected

variation in the level of resistance. The variance values were logged (base 10) to correct a right skew in the residuals.

The values plotted in Figure 5.6 were calculated as follows: the variance in resistance between clones taken from the same population was calculated. The mean variance was then calculated for each treatment, and the standard error in this variance. Finally, the mean variances and standard errors for treatments where the antibiotic environment was altered with the same frequency were averaged (taking the mean), to produce the values plotted in Figure 5.6.

5.3 Results

5.3.1 Responses of bacterial populations to heterogeneity in antibiotic exposure

To test how antibiotic heterogeneity affects the evolution of antibiotic resistance, we exposed bacterial populations to treatments where the antibiotics ceftriaxone and sulfamethoxazole were rotated at different rates. As a control, we also exposed bacterial populations to a constant concentration of either ceftriaxone or sulfamethoxazole (see Fig. 5.1). To measure the evolutionary response to antibiotics, we measured the growth rate of populations through time and estimated the rate of adaptation for each population (Fig. 5.2 A and B). To test the hypothesis that increasing antibiotic heterogeneity prevents the evolution of resistance, we tested for a negative correlation between the rate of antibiotic rotation and the rate of adaptation.

Contrary to our hypothesis, when we compared the cycling treatments to each other, increasing antibiotic heterogeneity actually accelerated the rate of adaptation to both ceftriaxone and sulfamethoxazole (Fig. 5.2 C and D. (For adaptation to ceftriaxone: $F_{3,278} = 56.2$ for overall F test, for specific treatment contrast t= -6.77 and $P < 0.001$. For adaptation to sulfamethoxazole: $F_{3,277} = 90.7$ for overall F test, for specific treatment contrast t = -9.15 and $P < 0.001$).

We also compared each cycling treatment to control populations that were constantly exposed to a single antibiotic. Rapid antibiotic cycling accelerated the rate of evolution relative to control populations that were constantly exposed to a single antibiotic. Specifically, we found that rapidly fluctuating exposure to ceftriaxone and sulfamethoxazole (antibiotic switched every day or two days) increased the rate of adaptation to ceftriaxone (Fig. 5.2. For one day fluctuations, $F_{5,308} = 23.7$ for the overall F test, for the specific treatment contrast t = 3.88 and $P < 0.001$. For two day fluctuations, $F_{5,308} = 23.7$ for the overall F test, for the specific treatment contrast t = 2.45 and $P < 0.05$). Rapid cycling (antibiotic switched every day) also accelerated adaptation to sulfamethoxazole ($F_{5,308} = 26.63$ for overall F test, for specific treatment contrast t = 4.47 and $P < 0.001$). The rate of adaptation to ceftriaxone of populations exposed to four day fluctuating treatments, and the rate of adaptation to sulfamethoxazole of populations exposed to two and four day fluctuating treatments, were not significantly different than the rates of adaptation of populations exposed to constant antibiotic treatments. These results highlight the risks of heterogeneous antibiotic treatments; populations exposed to antibiotic heterogeneity often adapted as well, or more quickly, than populations exposed to a single antibiotic treatment, even

though populations exposed to a heterogeneous treatment were only exposed to each antibiotic for half the number of days.

Note: A lower proportion of populations became extinct in the Chapter 5 experiments than in previous experiments (Chapters 3, 4). This is likely to be because populations were only exposed to one antibiotic at a time in Chapter 5, rather than combinations of antibiotics as in Chapters 3 and 4. In Chapter 4, when populations were exposed to ceftriaxone or sulfamethoxazole individually at concentrations similar to those used in Chapter 5, 3/44 or roughly 7% of populations became extinct. This is broadly similar to the population extinction rate of 2% in the Chapter 5 experiments.

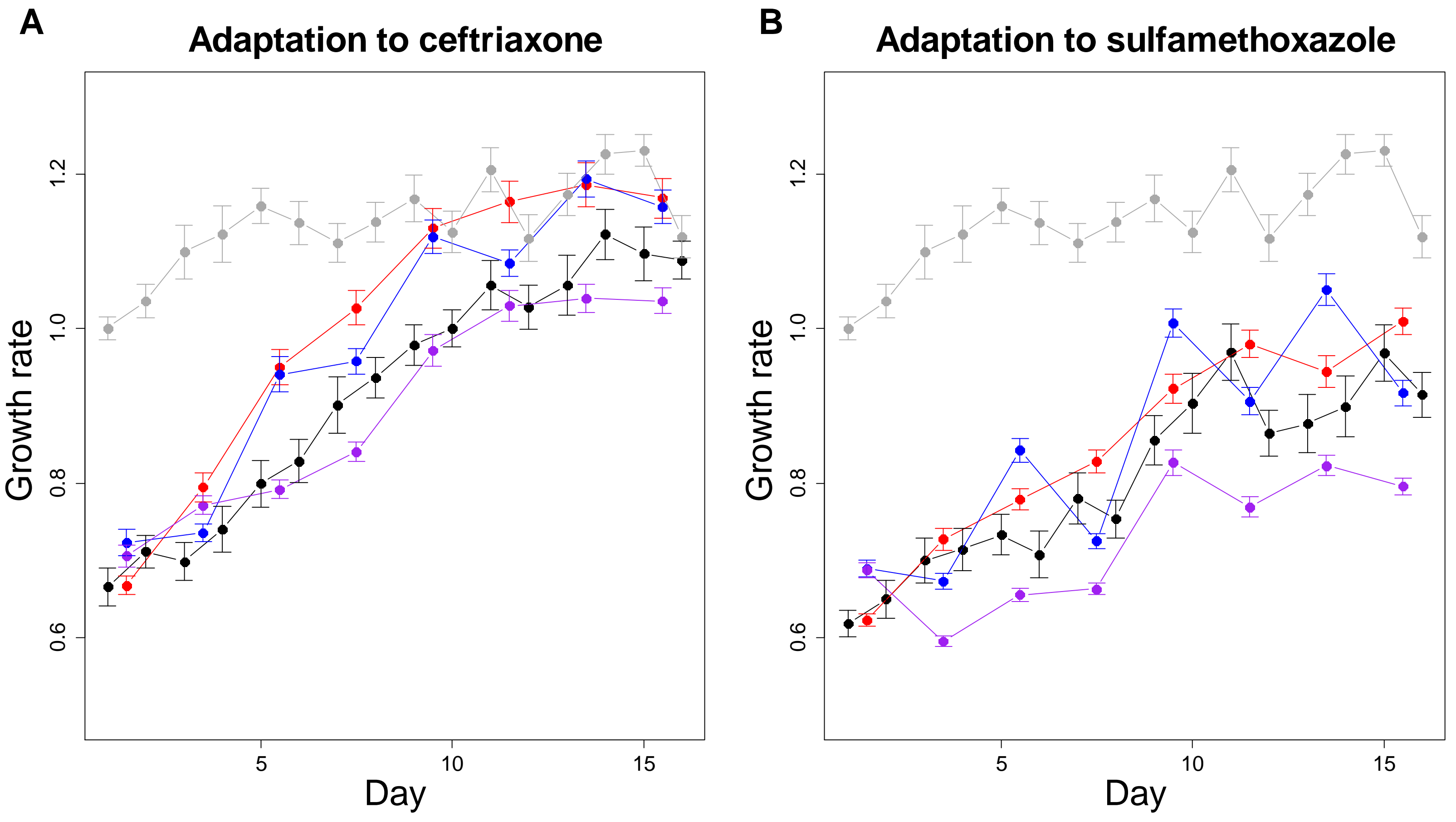


Figure 5.2. High levels of heterogeneity accelerate the evolution of antibiotic resistance. In panels *(A)* and *(B)*, red plotted points show the mean growth rate +/- s.e.m. on each day of populations assigned to the one day fluctuating treatment, blue shows populations exposed to the two day fluctuating treatment, purple shows populations exposed to the four day fluctuating treatment, black shows populations exposed to a constant treatment (either ceftriaxone or sulfamethoxazole), and grey represents the growth rate of a control evolved in antibiotic-free conditions. Each fluctuating treatment data point is an average across the two orders in which the antibiotics were applied for that treatment. Panel *(A)* shows adaptation to ceftriaxone, Panel *(B)* shows adaptation to sulfamethoxazole.

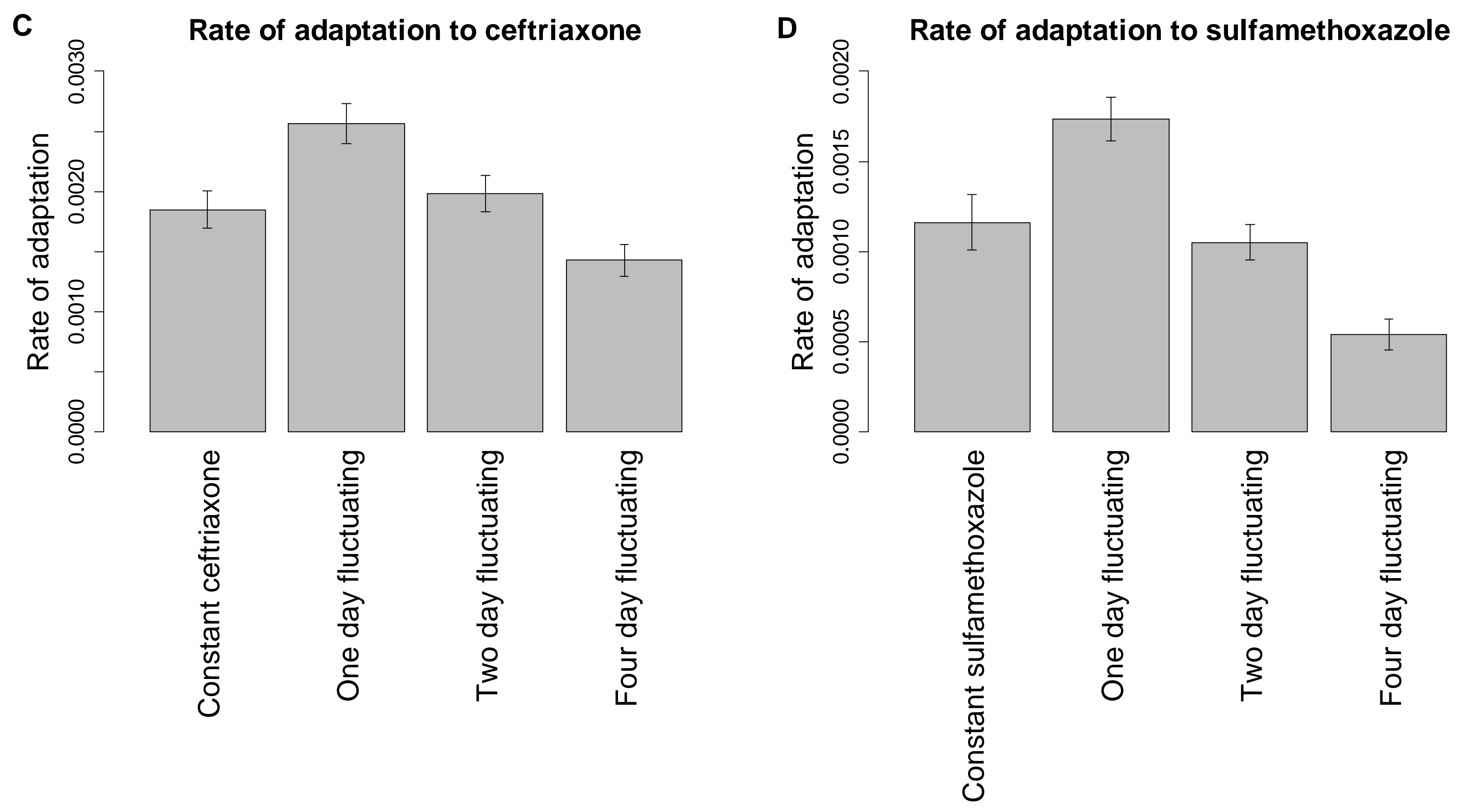


Figure 5.2 continued. Panels *(C)* and *(D)* show the mean rate of adaptation +/- s.e.m. to ceftriaxone and sulfamethoxazole respectively, of populations exposed to the constant antibiotic treatment and to each fluctuating treatment. Rate of adaptation is measured as change in growth rate per hour of active growth.

There was a positive correlation between rate of adaptation to ceftriaxone and rate of adaptation to sulfamethoxazole, so that populations which evolved more resistance to ceftriaxone also tended to evolve more resistance to sulfamethoxazole ($F_{4,277} = 93.64$ for overall F test, for specific treatment contrast $t = 7.24$, $P < 0.001$). Populations that were evolved with ceftriaxone adapted more rapidly than populations that were evolved with sulfamethoxazole (Fig. 5.3; $t_{62} = 3.12$, $P < 0.01$), suggesting that it is easier for *P. aeruginosa* populations to evolve ceftriaxone resistance than sulfamethoxazole resistance.

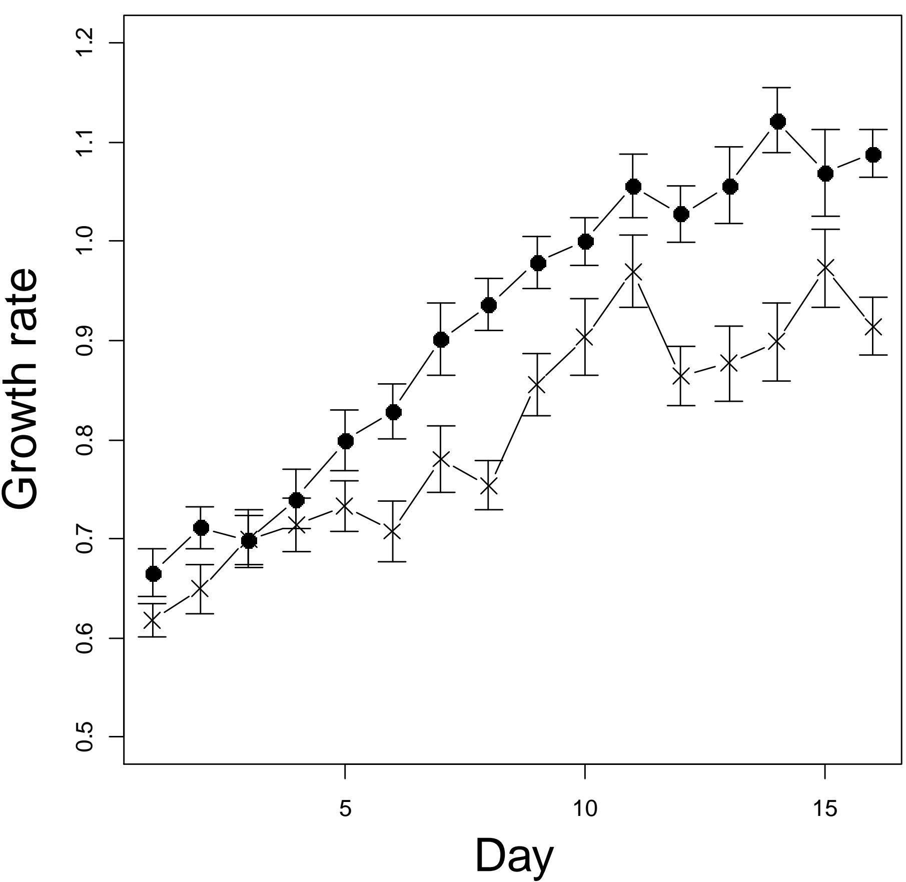


Figure 5.3. **Populations showed more adaptation to ceftriaxone than to sulfamethoxazole.** Filled circles show the mean growth rate +/- s.e.m. of populations exposed to the constant ceftriaxone treatment on each day of the selection experiment. Crosses are the growth rates of populations exposed to the constant sulfamethoxazole treatment on each day of the selection experiment.

5.3.2 Impact of environmental heterogeneity on the resistance of bacterial clones

To gain further insight into how antibiotic heterogeneity shapes the evolution of resistance, we measured the ceftriaxone and sulfamethoxazole resistance of individual clones that were isolated from populations exposed to antibiotic treatments or an antibiotic-free control environment, as well as the resistance of clones of the ancestral strain. To measure resistance, we calculated the growth rate of each clone in the presence of each antibiotic relative to its growth rate in antibiotic-free culture medium, such that a value of 1 means the antibiotic has no inhibitory effect on the growth of the clone. This approach provides a quantitative measure of resistance that can capture subtle differences in resistance while controlling for the potentially confounding effects of overall growth rate on resistance.

The ancestral strain had low resistance to the concentrations used of both ceftriaxone (resistance of 0.60; s.e. = 0.063) and sulfamethoxazole (resistance of 0.71; s.e. = 0.040). We found that increased resistance to both antibiotics evolved in all treatments, including antibiotic-free controls (Fig. 5.4; $t_{>5} > 2.5$, $P < 0.05$ for all treatments). This is likely to have been due to adaptation to laboratory conditions, in particular the bacterial clone used in this experiment had not previously been grown on Mueller Hinton 2 broth.

One possible explanation for the rapid adaptation of populations to both ceftriaxone and sulfamethoxazole is that cross-resistance occurs between these antibiotics. To test for cross-resistance, we compared the resistance of clones from populations evolved with a single antibiotic to the resistance of clones from control populations that evolved in

antibiotic-free conditions. We found that clones from populations evolved with ceftriaxone had equivalent levels of sulfamethoxazole resistance as clones from antibiotic-free populations (Fig. 5.4; $t_8 = 1.79$, $P = 0.11$). Similarly, clones from populations evolved with sulfamethoxazole had equivalent levels of ceftriaxone resistance as clones from antibiotic-free control populations ($t_8 = 1.69$, $P = 0.13$). Collectively, these results suggest that there is no overall tendency for cross-resistance between mechanisms that confer resistance to ceftriaxone and to sulfamethoxazole. However, we cannot rule out that if a larger sample size had been used, cross-resistance might have been detected. Genome sequencing of some of the clones would be an alternative way to look for cross-resistance in the system, to see whether clones contained mutations known to confer resistance to multiple antibiotics.

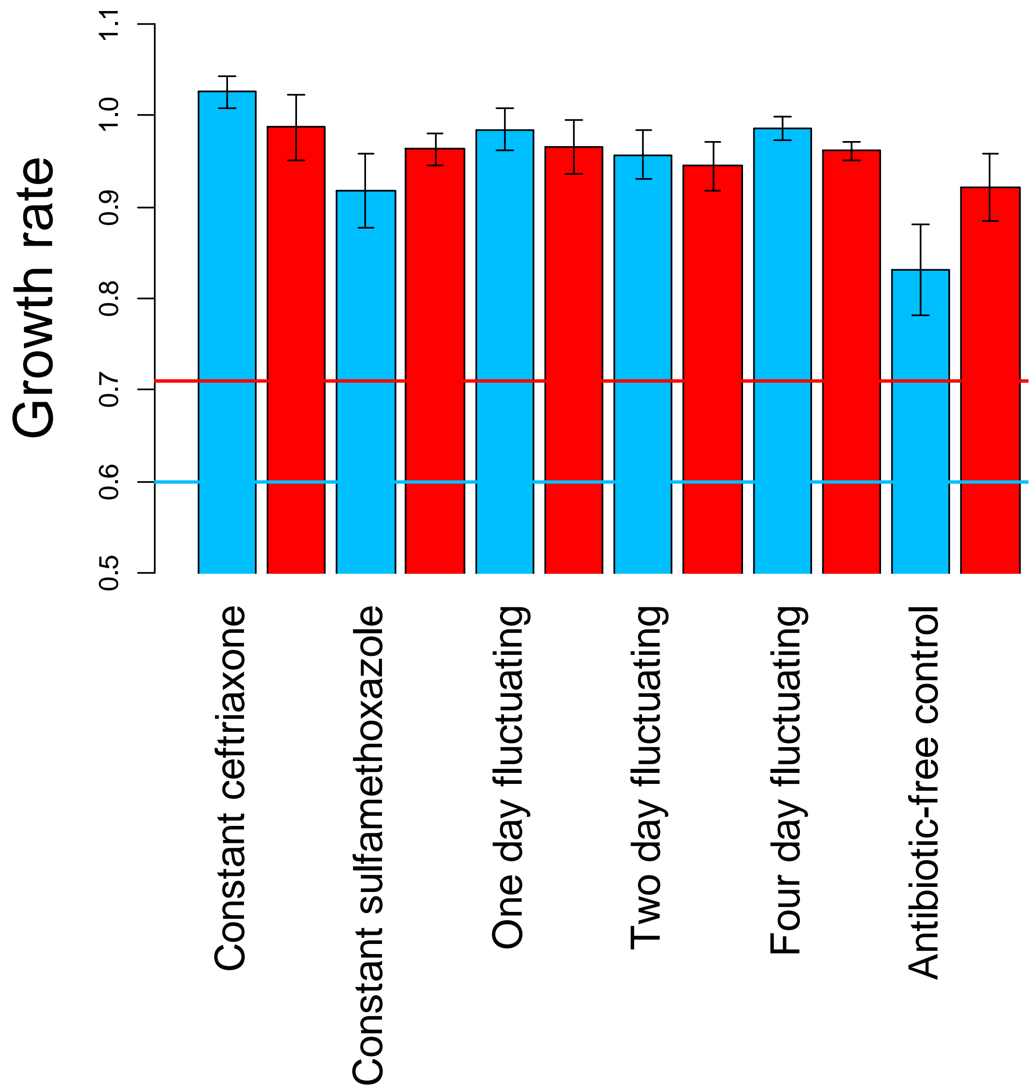


Figure 5.4. Growth rate of Day 16 clones when exposed to each antibiotic. Bars show the average growth rate +/- s.e.m. of populations evolved with each treatment when exposed to ceftriaxone (blue) and sulfamethoxazole (red). All growth rate values are calculated as the growth rate of a clone in a particular antibiotic environment relative to a growth rate of 1 of the same clone when assayed in antibiotic-free conditions. The blue horizontal line indicates the growth rate of the ancestral strain when exposed to ceftriaxone, and the red horizontal line the growth rate of the ancestral strain when exposed to sulfamethoxazole.

Note: the values plotted in Figure 5.4 were calculated as follows: I first calculated the mean growth rate for each population in each antibiotic by taking the mean across the clones for a particular population. The mean growth rate was then calculated for each treatment, and the standard error in this growth rate. Finally, the mean growth rate and standard errors for treatments where the antibiotic environment was altered with the same frequency were averaged (taking the mean), to produce the values plotted in Figure 5.4.

5.3.3 Testing for a cost of resistance

Environmental heterogeneity will be most effective at suppressing the evolution of resistance if resistance carries a cost. To test for the cost of antibiotic resistance, we compared the growth rate of populations adapted to each treatment when assayed in antibiotic-free conditions relative to the growth rate of a control evolved in antibiotic-free conditions when assayed in antibiotic-free conditions. The mean growth rate of clones adapted to each treatment when assayed in antibiotic-free conditions was not significantly different from the mean growth rate of clones evolved in the antibiotic-free environment, indicating that resistance did not carry a cost in our system (Fig. 5.5; $t_{39} < 1$, $P > 0.3$ for all treatments).

5.3.4 Fluctuating environments drive the evolution of diverse bacterial populations

To explain why higher levels of heterogeneity accelerated the evolution of antibiotic resistance, we hypothesised that heterogeneity could maintain genetic variation in the population on which selection could act. To test this idea, we estimated the genetic variation in ceftriaxone and sulfamethoxazole resistance as the variance in resistance between clones isolated from the same population. We found that there was more genetic variation between clones in the level of resistance to ceftriaxone and to sulfamethoxazole when fluctuations in the environment were more rapid (Fig. 5.6. For ceftriaxone: $F_{1,28} = 11.31$, $P < 0.01$. This was an overall F test. For sulfamethoxazole: $F_{1,28} = 5.43$,,$P < 0.05$. This was an overall F test).

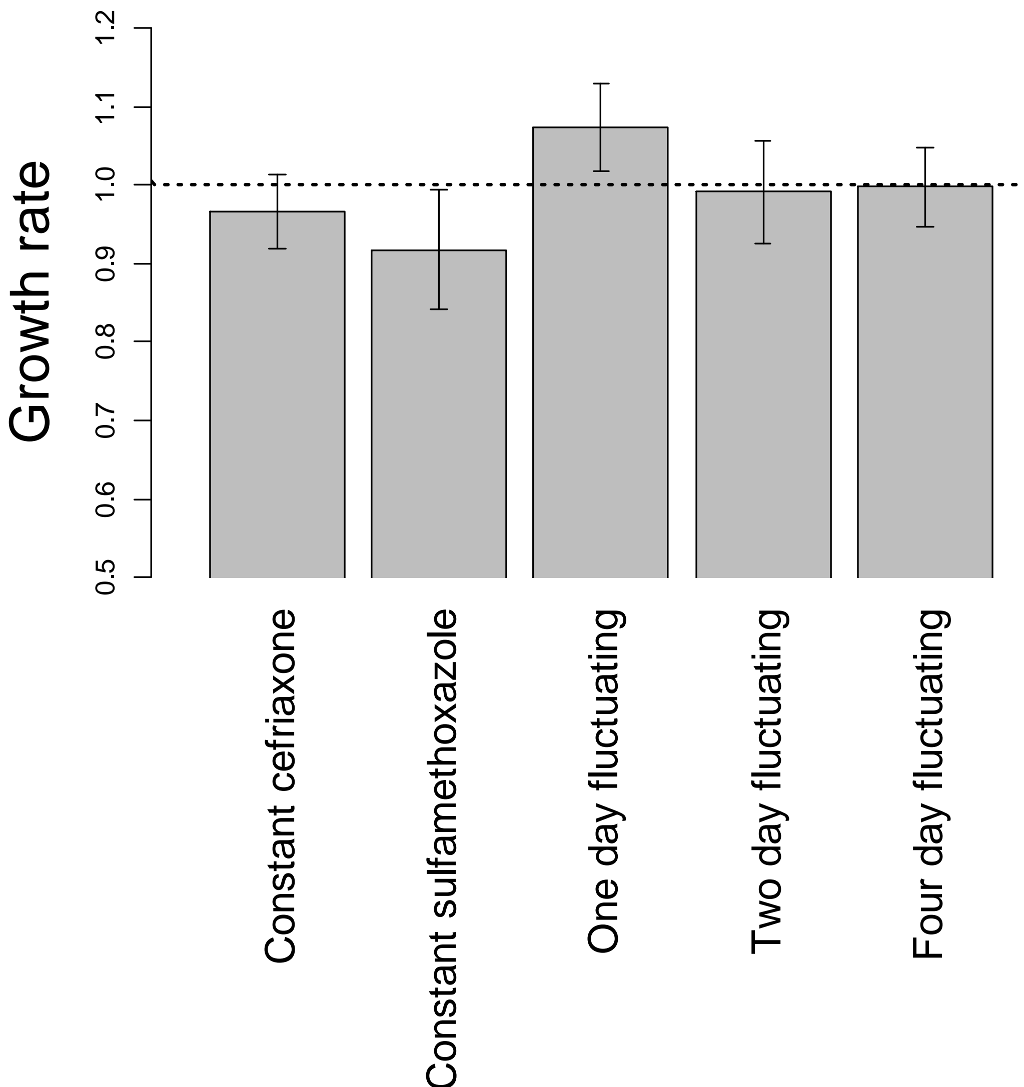


Figure 5.5. There was no cost of resistance in our system. Bars show the mean growth rate of populations evolved with each treatment when assayed in antibiotic-free conditions, relative to the growth rate of control populations evolved in antibiotic-free conditions, +/- s.e.m. The dashed line represents a growth rate of 1, where there is no cost of resistance: the growth rate of the bacteria evolved with antibiotics is equal to that of a control population, when both are grown in antibiotic-free conditions.

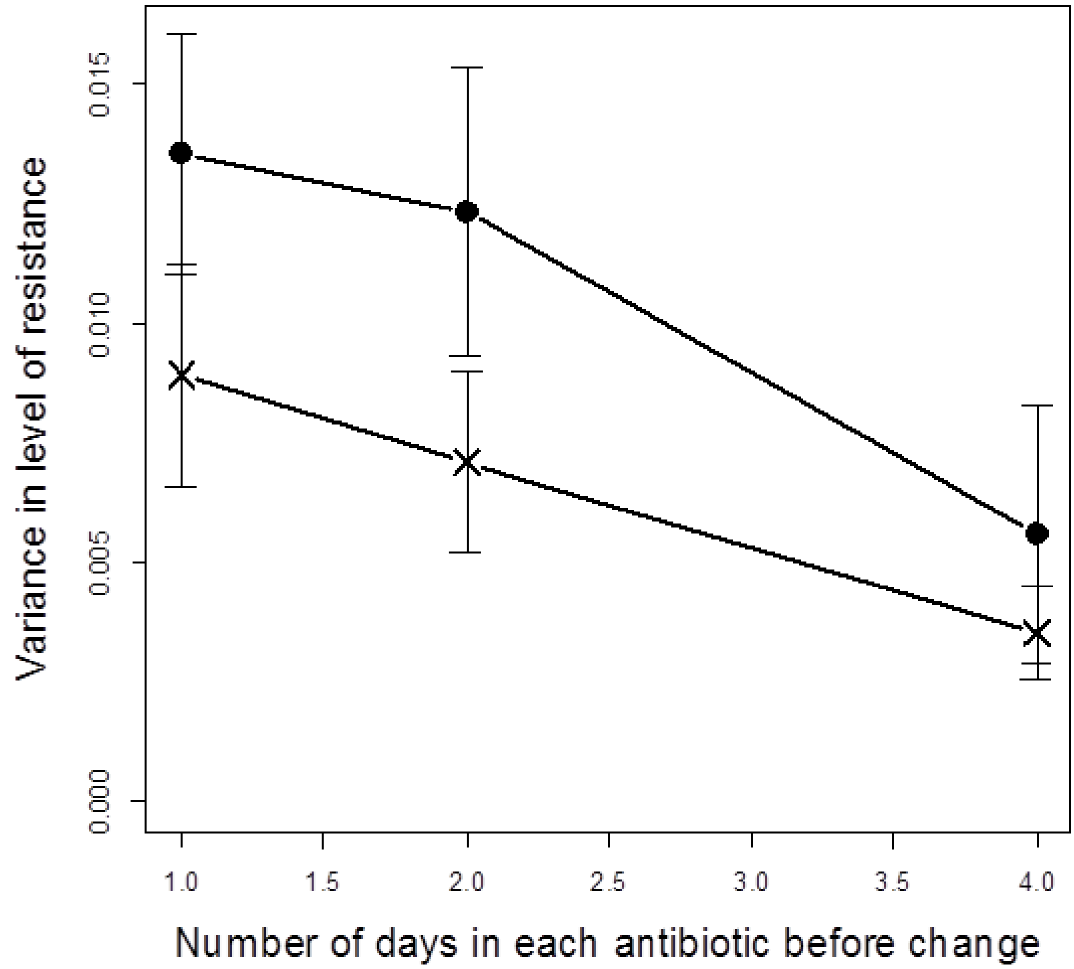


Figure 5.6. There was more variance in the level of resistance to each antibiotic when the level of heterogeneity was higher. Filled circles represent the mean variance in the level of resistance to ceftriaxone; crosses represent the mean variance in the level of resistance to sulfamethoxazole.

5.4 Discussion

To test how antibiotic heterogeneity affects the evolution of antibiotic resistance, we exposed *P. aeruginosa* to treatments where the antibiotics ceftriaxone and sulfamethoxazole were rotated at different rates in a laboratory setting. We found that

when heterogeneous treatments were compared, the more rapid the rotation of the antibiotics, the faster resistance evolved (see Fig. 5.2). We also found that rapid fluctuations in the antibiotics present led to faster evolution of resistance than constant exposure to one antibiotic. This is the first time, to our knowledge, that a laboratory-based study has been carried out comparing heterogeneous antibiotic environments with different rates of fluctuation to each other.

Mathematical models have predicted that an appropriate level of antibiotic heterogeneity will reduce the rate of evolution of antibiotic resistance compared to a constant antibiotic treatment [73, 140], as have experimental evolution studies [148, 149]. Clinical studies however have not found conclusive evidence for the advantage of heterogeneity [15, 70, 138]. Here, we find that rapid fluctuations in the antibiotic environment actually accelerate the evolution of resistance to a particular antibiotic compared to constant exposure to the same antibiotic (see Fig. 5.2). The result is quite marked given that populations exposed to a fluctuating treatment had only half as many days of exposure to a particular antibiotic as populations exposed to the constant treatment, yet in some cases adapted more quickly than populations exposed to the constant treatment. The result is however consistent with previous work which found that bacterial populations adapted to fluctuating low and high quality environments had as high a growth rate in either environment as populations which had adapted to one environment for twice as long [150]. Proponents of heterogeneity suggest that resistance to one antibiotic will decline when a population is exposed to a different antibiotic instead, as the selection pressure for resistance to the first antibiotic will be removed when the population is not exposed to that antibiotic. There may also be trade-offs between resistance to different antibiotics [82, 151]. We find, however, that

adaptation to one antibiotic drives adaptation to the other antibiotic. Therefore, the selection pressure for resistance to ceftriaxone is maintained when populations are exposed to sulfamethoxazole, and vice versa.

Heterogeneity works best when resistance to a particular antibiotic carries a cost in the absence of that antibiotic. Sensitive bacteria can then out-compete resistant bacteria in the absence of the antibiotic, reducing the frequency of resistant bacteria or even driving the resistant bacteria to extinction. The cost of resistance to an antibiotic can vary greatly, but tends to be much smaller than the benefit of resistance in the presence of the antibiotic, so that resistance declines less quickly in the absence of the antibiotic than it increases in the presence of it [33, 152]. Looking at the mean values for each treatment, none of our resistant bacteria showed a cost of resistance on Day 16 of the experiment, which would remove the selective advantage of sensitive strains in the absence of a particular antibiotic. We cannot rule out, however, that resistance may have been costly at earlier time points in the experiment but bacteria had compensated for the cost of resistance by Day 16.

When the heterogeneous treatments are compared, we find that the higher the level of heterogeneity, the faster antibiotic resistance evolves (see Fig. 5.2). This result is in contrast to most of the published mathematical models which predict that evolution of resistance will be delayed with increasing levels of heterogeneity [73, 140]. We explain our result as follows: higher levels of heterogeneity produced greater variation in the level of resistance in our experiment (see Fig. 5.6), suggesting that there was greater genetic diversity in the higher heterogeneity treatments than the lower heterogeneity

treatments. When exposed to the same antibiotic for an extended period of time, selective sweeps for mutations conferring resistance to that antibiotic will reduce variation in the population. In contrast, when changes in the antibiotic present are more frequent, there will be less time for selective sweeps to occur before the selection pressures are altered. By maintaining variation in the population, there is more diversity for selection to act on over an extended course of evolution and more evolutionary trajectories are available. This explanation is consistent with other studies which show that environmental heterogeneity can maintain diversity in bacterial populations [153, 154]. Once a population starts to adapt more quickly to the environment, the population size of resistant bacteria will be larger, further increasing the supply rate of beneficial mutations and the probability that beneficial mutations will arise within the resistant background, rather than in a sensitive background generating clonal interference.

Our results highlight that the choice of antibiotics to be used in a heterogeneous treatment may be important in determining the effectiveness of the treatment (see also [138]). Similar work has also been carried out looking at herbicide resistance in weeds. Lagator *et al.* carried out a study looking at how herbicide cycling affected the evolution of herbicide resistance in *Chlamydomonas reinhardtii*, and found that cycling could prevent, have no impact on or even accelerate the evolution of herbicide resistance depending on the herbicides used [155].

An important difference between our work and clinical scenarios is in the severity of the bottlenecking bacteria undergo. While we bottlenecked our populations down to approximately 10^5 cells every 24 hours, in clinical situations the number of cells

colonising new patients to found new infections may be much smaller, representing a much more severe bottleneck and causing loss of diversity in the population. Supporting the importance of population size in determining outcomes, Kouyos *et al.* show that in their mathematical model, heterogeneity is successful in limiting the prevalence of antibiotic resistance only when population size is small, perhaps because extinction events impede the development of resistance in small populations [146].

To our knowledge, this is the first time that the effect of varying levels of heterogeneity within heterogeneous environments on the rate of evolution of antibiotic resistance has been tested experimentally *in vitro*. In contrast to the predictions of the majority of mathematical models, we find that the rate of evolution of resistance increases with increasing environmental heterogeneity. The effect of high levels of heterogeneity in maintaining genetic diversity have not generally been considered in the mathematical models, but may be important in allowing for continued variation on which selection can act.

Chapter 6

6 General Discussion

In this thesis I use experimental evolution in *P. aeruginosa* to test hypotheses about how multiple-antibiotic treatments affect the rate of evolution of antibiotic resistance. Specifically, I look at combination treatments and at treatments which create heterogeneity in the antibiotic environment. Here, I discuss the overall findings emerging from my research, the limitations of the research and possible extensions.

6.1 Themes emerging from my research

6.1.1 The selection pressure imposed by antibiotics on bacterial populations determines the rate of evolution of resistance

The evolution of antibiotic resistance is a classic example of Darwinian selection. The greater the selective advantage of resistant strains over sensitive strains, the faster resistance evolves. In Chapters 3, 4 and 5, I create treatments with varying inhibitory effects on the sensitive wild type strain. I consistently find that the greater the inhibitory effect of the treatment on the sensitive strain, the faster resistance evolves. On a broader scale, analyses of clinical data have found a strong correlation between the quantity of antibiotic used (i.e. the strength of the selection pressure for resistance) and the amount

of resistance amongst bacterial populations, both on an individual patient level [156, 157] and at a hospital ward [158] or community [19, 159, 160] level.

It is a well established principle that a stronger selection pressure will increase the rate of evolution, and some clinical strategies have the aim of reducing the strength of the antibiotic selection pressure imposed on bacterial populations (for example, the drive to prescribe antibiotics only when they are really needed). Clinical strategies based on other principles are also used, for example using combinations of antibiotics in an attempt to prevent the evolution of resistance. My work suggests that strategies based on reducing the strength of the antibiotic selection pressure are of primary importance in reducing the evolution of resistance. Thus, there is a trade-off where clinicians must balance immediate benefit to a patient of antibiotic treatment with preserving the usefulness of an antibiotic for future patients. However, there are cases where antibiotic use can be reduced without detriment to a patient, for example where a patient is not infected with a bacterial pathogen. In addition, recent studies have shown that shorter courses of antibiotics can be as effective as longer courses in curing patients, while reducing the evolution of resistance (see [161] for a discussion of this topic).

In Chapter 1 of this thesis, I set out that, prior to beginning experimental work, it was unclear how important the supply rate of resistance-conferring mutations would be in determining the rate of evolution of antibiotic resistance, compared to the strength of selection increasing the frequency of resistance mutations once they had arisen in the population. It was therefore unclear whether more inhibitory antibiotic combinations would increase or decrease the rate of evolution of resistance compared to less

inhibitory combinations. More inhibitory combinations would lower population sizes, reducing the supply rate of resistance conferring-mutations, but would also increase the selective advantage of such mutations once they arose in the population. In Chapters 3 and 4 of this thesis, I found that the more inhibitory an antibiotic combination to sensitive ancestral bacteria, the more quickly resistance evolved (se Figs. 3.4B and 4.3A), suggesting that the supply of resistance-conferring mutations did not limit the rate of evolution of resistance, and that the greater selective advantage of resistant mutants when combinations were more inhibitory was the dominant factor determining the rate of evolution of resistance. The results presented in Chapter 5 do not provide a clear indication as to whether mutation rate was limiting the rate of evolution of resistance in this experiment.

6.1.2 Synergistic combinations do not accelerate the evolution of antibiotic resistance

There has been concern in the evolution literature in recent years that synergistic combinations of antibiotics, favoured by clinicians for their enhanced effectiveness for a given concentration of antibiotics, actually accelerate the evolution of antibiotic resistance (see for example [56, 68, 103, 113, 137]). These concerns are based on a hypothesis proposed by Hegreness *et al.* [68], which has limited experimental support [68, 113]. In Chapter 3, I test this hypothesis on the broadest experimental scale attempted so far, and for the first time produce experimental data refuting the idea that clinicians should be concerned about the use of synergistic combinations. I find that synergistic combinations do accelerate the evolution of antibiotic resistance, but only because they produce greater inhibition of the sensitive strain. I do not find any

evidence for Hegreness *et al.*'s proposal that synergistic combinations accelerate the evolution of resistance compared to antagonistic combinations even when the inhibitory effect of both combinations on the sensitive strain is equal. Thus, the choice of a synergistic combination should be seen in the broader context of the use of a more inhibitory antibiotic treatment, and a possible trade-off between providing the most effective treatment for a patient now and preserving the effectiveness of the antibiotics for future patients, rather than a specific concern about the use of synergistic combinations.

6.1.3 Antibiotic heterogeneity may conserve variation on which selection can act and so may have unexpected effects on the evolution of antibiotic resistance

In Chapter 5, I test, for the first time in a laboratory-based study, how the level of antibiotic heterogeneity bacteria are exposed to affects the rate of evolution of antibiotic resistance. In contrast to mathematical models exploring this topic [73, 140], I find that the greater the antibiotic heterogeneity in the environment, the faster antibiotic resistance evolves. I also find that higher levels of heterogeneity produce greater variation in the level of resistance, suggesting that there is greater genetic diversity in populations exposed to higher heterogeneity treatments than lower heterogeneity treatments. It may be that in the low heterogeneity treatments, selective sweeps reduce variation in the population, but that the frequent altering of the direction of selection in high heterogeneity treatments preserves variation. Thus, there is more diversity for selection to act upon in the higher heterogeneity treatments, accelerating the evolution of resistance. The role of antibiotic heterogeneity in maintaining variation in

populations has not generally been considered in mathematical models, but may be important in determining the evolution of antibiotic resistance.

6.2 Limitations of my research

As discussed in Chapter 1, all the antibiotics were used at concentrations below the MIC for the wild type sensitive strain. This approach was necessary in order to quantify interactions between antibiotics for Chapters 2 and 3, but meant that the antibiotic concentrations used were well below those aimed for clinically. When antibiotic concentrations are above the MIC for the wild type sensitive strain, the sensitive strain will not survive and so the selection coefficient in favour of resistant strains is 1. At these concentrations, increasing the antibiotic concentration will decrease the number of resistant strains able to survive and so may slow the rate of evolution of resistance. Hegreness *et al.*'s hypothesis that synergistic interactions accelerate the evolution of antibiotic resistance [68], as discussed in Chapter 3, is applicable only to situations where the sensitive wild type strain can grow, if only slowly. However, it is thought that resistance is more likely to evolve in clinical situations where the antibiotic concentration is lower than was aimed for; for example in body tissues which the antibiotic does not penetrate well [84, 162]. In these low antibiotic concentration refuges, mutants with a low level of antibiotic resistance, and wild type sensitive cells, may survive and evolve higher level antibiotic resistance.

My work was carried out *in vitro* in a simple experimental system. While this had a number of advantages as discussed in Chapter 1, my system could not capture the full complexity of a clinical situation. For example, the immune system may moderate the

effects I found in my system, and my system did not capture varying antibiotic concentrations across different tissues in the body. The applicability of my results to clinical situations should therefore be treated with caution. Another important difference between evolution in my system and a clinical scenario is that I studied only evolution from *de novo* mutation. In clinical situations, resistance is often conferred by plasmids. These plasmids commonly carry several resistance genes, and so take-up of one plasmid may transform a clone from being antibiotic sensitive to multiple-antibiotic resistant [33]. Antibiotic synergy may be less common when bacteria are multiple-antibiotic resistant [135], and an antibiotic heterogeneity strategy relies on bacteria only acquiring resistance to one antibiotic at a time. The frequency of horizontal gene transfer and therefore the possible importance of this limitation differs between bacterial species; *M. tuberculosis* usually evolves antibiotic resistance through point mutations [33], and *P. aeruginosa* readily evolves resistance *de novo*, even within the course of an infection of a single patient [158], but for some other species plasmid-mediated resistance is a crucial part of their resistance [163].

6.3 Further work to extend my research

My work on antibiotic heterogeneity, described in Chapter 5, could be extended to explore further how different levels of genetic diversity may have affected the evolution of resistance. Clones could be taken from populations exposed to different treatments, and genome sequencing used to test the hypothesis that populations exposed to higher heterogeneity treatments maintained more genetic variation in the population. If divergence between different strains in a population happens soon after first treatment

with a new antibiotic, we might expect that high heterogeneity treatments would show longer branch lengths, on average, than low heterogeneity treatments, indicating that divergence between strains within a population is greater in high heterogeneity treatments. In addition, it would be interesting to test whether the mutations selected were single-antibiotic resistance mutations or multiple-antibiotic resistance mutations.

In Chapter 5, adaptation to either ceftriaxone or sulfamethoxazole appeared to drive adaptation to the other antibiotic as well. This result explains why populations exposed to heterogeneous treatments evolved resistance at least as quickly as populations exposed to constant antibiotic treatments, but does not explain why higher levels of heterogeneity accelerated the evolution of resistance relative to lower levels of heterogeneity. It would be interesting to check whether this result held for other antibiotic combinations, especially combinations which do not produce positive cross-resistance.

Chapter 7

7 General Conclusions

In this thesis, I investigate how aspects of multiple-antibiotic treatments impact the rate of evolution of antibiotic resistance in the opportunistic human pathogen *Pseudomonas aeruginosa.* In particular, I look at the impact of interactions between antibiotics in combination on the evolution of resistance, and how creating heterogeneity in the antibiotic environment by rotating the antibiotics used may change the rate of evolution of resistance.

In Chapter 2, I characterise the interactions among 120 antibiotic combinations. I find that the antibiotic families in combination (i.e. the mechanisms of action of the antibiotics) predict the type of interaction. I also find that when two bactericidal antibiotics interact, interactions tend to be synergistic, whereas when two bacteriostatic antibiotics interact, interactions tend to be antagonistic. In Chapter 3, I take a subset of these combinations and test, on the largest scale attempted so far, the influential but weakly-evidenced hypothesis that synergistic combinations of antibiotics accelerate the evolution of resistance. Once the initial inhibitory effect of each antibiotic combination is controlled for, I find that the type of interaction does not influence the rate of evolution of resistance. In Chapter 4, I test the hypothesis that resistance evolves more slowly to a combination of the antibiotics ceftriaxone and sulfamethoxazole than would

be predicted by the initial inhibitory effect of these antibiotics, but find no evidence to support this hypothesis.

In Chapter 5 I test, for the first time in a laboratory setting, how varying the rate of fluctuation in the antibiotics present in a heterogeneous antibiotic environment impacts the rate of evolution of resistance. In contrast to the majority of published mathematical models, I find that the rate of evolution of resistance increases with increasing levels of antibiotic heterogeneity. I propose that this is because higher levels of heterogeneity maintain more variation in the population on which selection can act.

Appendix A: Supplementary Information

Validation of growth rate measurements used: based on Chapter 3 data

To validate the use of BacTiter-Glo (Promega) values taken after 24 hours of growth as a measure of bacterial growth rate, we also estimated the exponential growth rate of each population on Day 1 of the Chapter 3 selection experiment (see Chapter 3 Materials and Methods), and the peak luminescence values reached on Day 1. There were strong correlations between growth rate estimated using $\log_{10}$ BacTiter-Glo (Promega) and growth rate estimated using exponential growth or $\log_{10}$ peak luminescence (Fig. S1; $t_{13} = 4.30$, $P < 0.001$ for correlation between BacTiter-Glo values and exponential growth rates, and $t_{13} = 11.6$, $P < 0.001$ for correlation between BacTiter-Glo values and peak luminescence values), indicating that BacTiter-Glo (Promega) can reliably be used as a proxy for bacterial growth rate. Exponential growth rate was not considered to be a reliable growth rate measure over the course of the selection experiment as populations may alter their level of *lux* expression during adaptation.

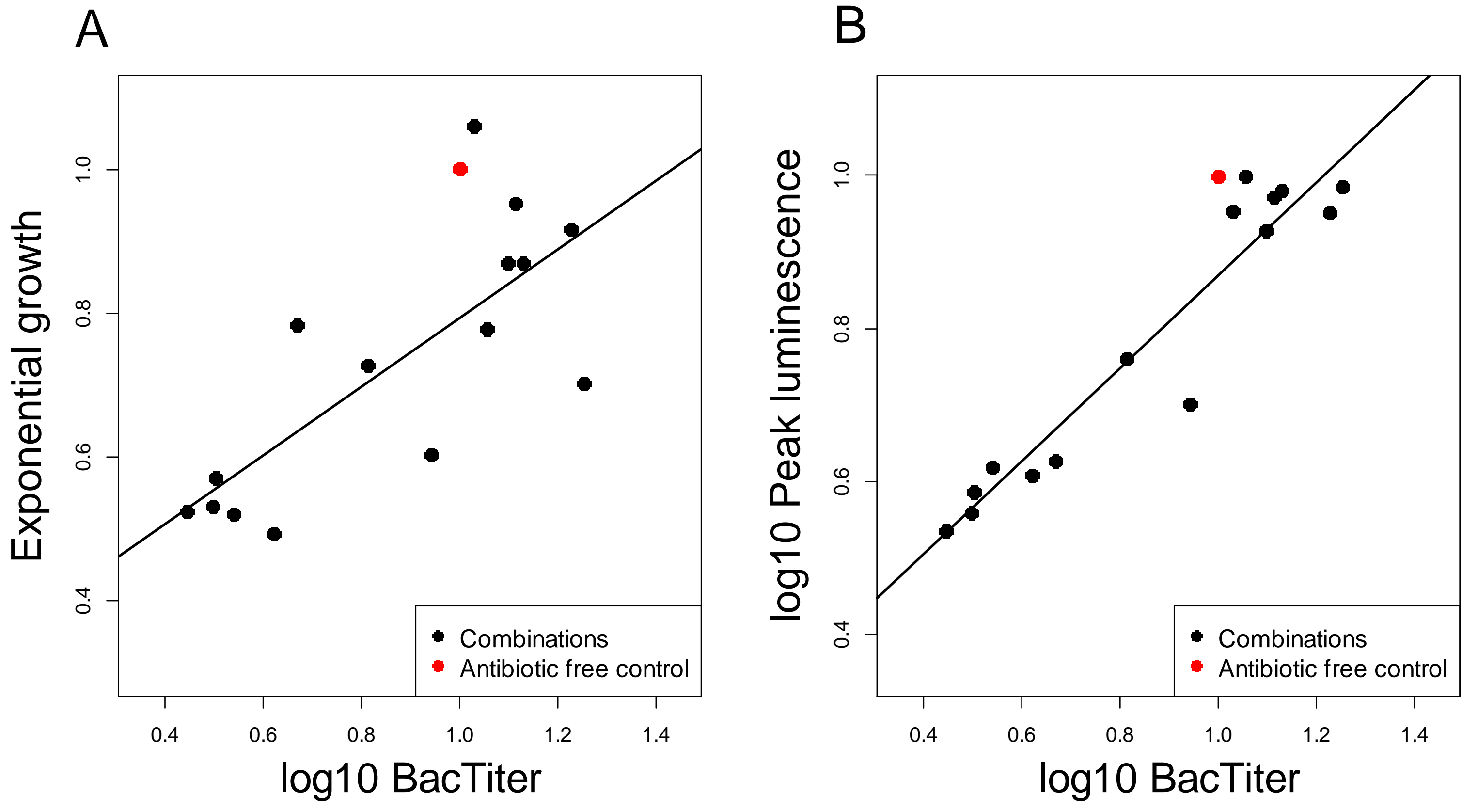


Figure S1. There were strong correlations between growth rate estimated using $\log_{10}$ BacTiter-Glo (Promega) and using exponential growth and $\log_{10}$ peak luminescence, based on Chapter 3 data. There was a positive correlation between growth rate measured by BacTiter-Glo (Promega) and growth rate measured using exponential growth (*A*). There was also a positive correlation between growth rate measured by BacTiter-Glo (Promega) and growth rate measured using peak luminescence (*B*). Growth rate values are standardised relative to growth rate of the antibiotic-free control of 1. Black dots represent the mean value for each antibiotic combination; the red dots represent the mean value for a control grown in antibiotic-free conditions. Black lines are the best fits to the antibiotic combinations (black dots).

Calculating the rate of adaptation

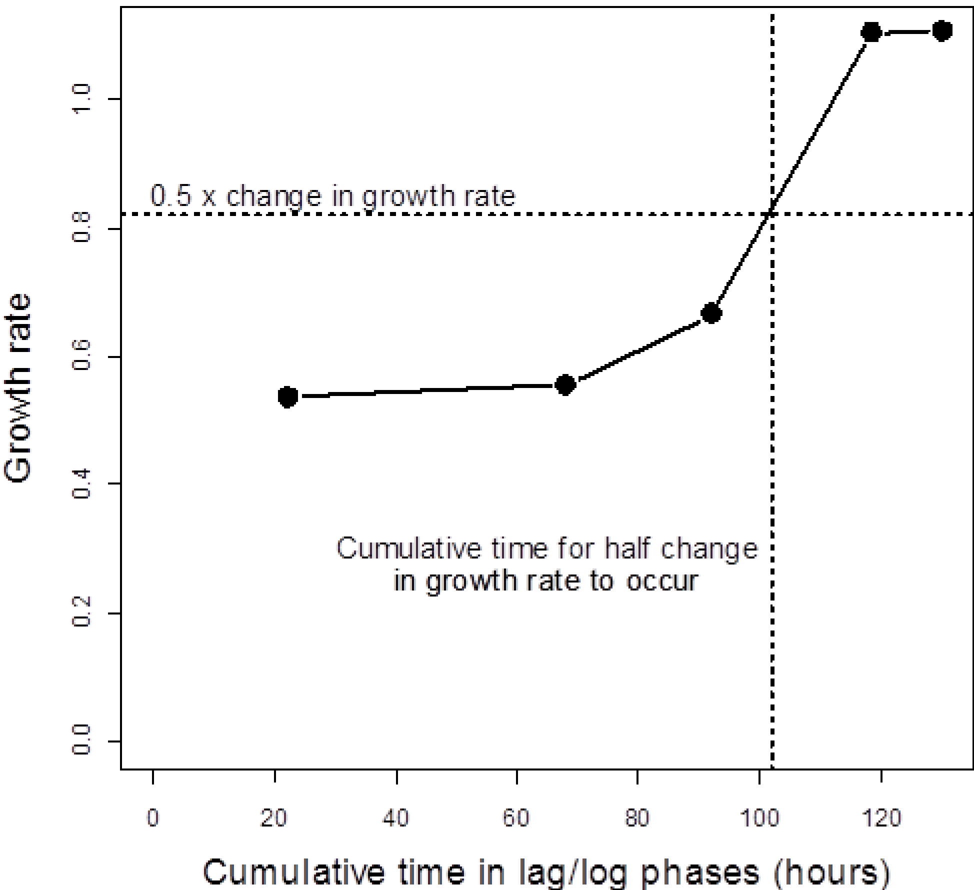


Figure S2. Illustrative example of how rate of adaptation was calculated in Chapters 3, 4 and 5. First 0.5 x the change in growth rate of the population during the course of selection was calculated. Then the cumulative time bacteria spent actively growing (in lag or log phases) to achieve 0.5 x the change in growth rate was calculated using the *approx* function in R 2.13.2 [131]. Rate of adaptation was calculated as 0.5 x change in growth rate/ cumulative time taken to achieve that change in growth rate.

Appendix B: Plate layouts

Chapter 2 treatment plate layout

The plate layout overleaf shows which treatment was placed in each well for the experiment determining interactions between antibiotics in Chapter 2. The key to the plate layout is as follows:

Blue shading- combination treatment

Grey shading- individual antibiotic treatment

Green shading- antibiotic-free control treatment

Purple shading- blank wells with no bacterial culture. These blanks were used to monitor levels of background noise in the luminescence reading. These wells were transferred to fresh plates in the same way as the wells containing bacterial cultures, to detect if the transfer process was introducing contamination to cultures.

Orange shading- these wells were inoculated with saturated bacterial culture and used for calibrating the spectrometer only.

Code letter	**Antibiotic**
A	Cefsulodin
B	Cefotaxime
C	Ceftriaxone
D	Piperacillin
E	Meropenem
F	Carbenicillin
G	Polymyxin B
H	Kanamycin
I	Tobramycin
J	Amikacin
K	Ciprofloxacin
L	Enoxacin
M	Levofloxacin
N	Mafenide
O	Sulfamethoxazole
P	Rifampicin

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	AB	AJ	BD	BL	CG	CO	DK	EH	EP	FN	GM	HM	IN	JP	LO	A	C	E	G	I	K	M	O	
B	AB	AJ	BD	BL	CG	CO	DK	EH	EP	FN	GM	HM	IN	JP	LO	A	C	E	G	I	K	M	O	
C	AC	AK	BE	BM	CH	CP	DL	EI	FG	FO	GN	HN	IO	KL	LP	A	C	E	G	I	K	M	O	
D	AC	AK	BE	BM	CH	CP	DL	EI	FG	FO	GN	HN	IO	KL	LP	A	C	E	G	I	K	M	O	
E	AD	AL	BF	BN	CI	DE	DM	EJ	FH	FP	GO	HO	IP	KM	MN	A	C	E	G	I	K	M	O	
F	AD	AL	BF	BN	CI	DE	DM	EJ	FH	FP	GO	HO	IP	KM	MN	A	C	E	G	I	K	M	O	
G	AE	AM	BG	BO	CJ	DF	DN	EK	FI	GH	GP	HP	JK	KN	MO	B	D	F	H	J	L	N	P	
H	AE	AM	BG	BO	CJ	DF	DN	EK	FI	GH	GP	HP	JK	KN	MO	B	D	F	H	J	L	N	P	
I	AF	AN	BH	BP	CK	DG	DO	EL	FJ	GI	HI	IJ	JL	KO	MP	B	D	F	H	J	L	N	P	
J	AF	AN	BH	BP	CK	DG	DO	EL	FJ	GI	HI	IJ	JL	KO	MP	B	D	F	H	J	L	N	P	
K	AG	AO	BI	CD	CL	DH	DP	EM	FK	GJ	HJ	IK	JM	KP	NO	B	D	F	H	J	L	N	P	
L	AG	AO	BI	CD	CL	DH	DP	EM	FK	GJ	HJ	IK	JM	KP	NO	B	D	F	H	J	L	N	P	
M	AH	AP	BJ	CE	CM	DI	EF	EN	FL	GK	HK	IL	JN	LM	NP									
N	AH	AP	BJ	CE	CM	DI	EF	EN	FL	GK	HK	IL	JN	LM	NP									
O	AI	BC	BK	CF	CN	DJ	EG	EO	FM	GL	HL	IM	JO	LN	OP									
P	AI	BC	BK	CF	CN	DJ	EG	EO	FM	GL	HL	IM	JO	LN	OP									

Chapter 3 treatment plate layout

The plate layout overleaf shows which treatment was placed in each well for the selection experiment described in Chapter 3. The key to the plate layout is as follows:

Blue shading- combination treatment

Grey shading- individual antibiotic treatment

Green shading- antibiotic-free control treatment

Purple shading- blank wells with no bacterial culture. These blanks were used to monitor levels of background noise in the luminescence reading. These wells were transferred to fresh plates in the same way as the wells containing bacterial cultures, to detect if the transfer process was introducing contamination to cultures.

Orange shading- these wells were inoculated with saturated bacterial culture and used for calibrating the spectrometer only.

Black shading- wells containing treatments which were not used in any analysis (e.g. because the treatment was an individual antibiotic that was not a β-lactam, aminoglycoside or quinolone antibiotic).

Combination treatments- Code letter	Antibiotic combination
A	Amikacin - levofloxacin
B	Amikacin - sulfamethoxazole
C	Amikacin - rifampicin
D	Amikacin - ceftriaxone
E	Amikacin - polymyxin B
F	Levofloxacin - sulfamethoxazole
G	Levofloxacin - rifampicin
H	Levofloxacin - ceftriaxone
I	Levofloxacin - polymyxin B
J	Sulfamethoxazole - rifampicin
K	Sulfamethoxazole - ceftriaxone
L	Sulfamethoxazole - polymyxin B
M	Rifampicin - ceftriaxone
N	Rifampicin - polymyxin B
O	Ceftriaxone - polymyxin B

Individual treatments- Code number	**Antibiotic**
2	Cefotaxime
3	Ceftriaxone
4	Piperacillin
6	Carbenicillin
7	Polymyxin B
8	Kanamycin
9	Tobramycin
10	Amikacin
11	Ciprofloxacin
13	Levofloxacin
15	Sulfamethoxazole
16	Rifampicin
18	Ceftazidime
21	Pefloxacin
22	Sparfloxacin

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	A		10	4			8		
B	B	C	D	E	F	G	H	I	J	K	L	M	N	O	A	B		11		21	15	9		
C	C	D	E	F	G	H	I	J	K	L	M	N	O	A	B	C			6	22	16	10		
D	D	E	F	G	H	I	J	K	L	M	N	O	A	B	C	D		13	7			11		
E	E	F	G	H	I	J	K	L	M	N	O	A	B	C	D	E			8	2	18			
F	F	G	H	I	J	K	L	M	N	O	A	B	C	D	E	F		15	9	3		13		
G	G	H	I	J	K	L	M	N	O	A	B	C	D	E	F	G		16	10	4				
H	H	I	J	K	L	M	N	O	A	B	C	D	E	F	G	H			11		21	15		
I	I	J	K	L	M	N	O	A	B	C	D	E	F	G	H	I	2	18		6	22	16		
J	J	K	L	M	N	O	A	B	C	D	E	F	G	H	I	J	3		13	7				
K	K	L	M	N	O	A	B	C	D	E	F	G	H	I	J	K	4			8	2	18		
L	L	M	N	O	A	B	C	D	E	F	G	H	I	J	K	L		21	15	9	3			
M	M	N	O	A	B	C	D	E	F	G	H	I	J	K	L	M	6	22	16	10	4			
N	N	O	A	B	C	D	E	F	G	H	I	J	K	L	M	N	7			11		21		
O	O	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	8	2	18		6	22		
P	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O		9	3		13	7			

Chapter 4 treatment plate layout

The plate layout overleaf shows which treatment was placed in each well for the selection experiment described in Chapter 4. The key to the plate layout is as follows:

Blue shading- combination treatment

Grey shading- individual antibiotic treatment

Green shading- antibiotic-free control treatment

Purple shading- blank wells with no bacterial culture. These blanks were used to monitor levels of background noise in the luminescence reading. These wells were transferred to fresh plates in the same way as the wells containing bacterial cultures, to detect if the transfer process was introducing contamination to cultures.

Orange shading- these wells were inoculated with saturated bacterial culture and used for calibrating the spectrometer only.

The first number in each double-digit code represents the concentration of ceftriaxone in the treatment (none, low, medium or high represented by 0, 1, 2 or 3 respectively). The second number in each double-digit code represents the concentration of sulfamethoxazole in the treatment. This is shown in the table overleaf.

		Sulfamethoxazole concentration			
Ceftriaxone concentration		None	Low	Medium	High
	None	00	01	02	03
	Low	10	11	12	13
	Medium	20	21	22	23
	High	30	31	32	33

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	00	10	20	30	01	11	21	31	02	12	22	32	03	13	23	33	00	01	02	03	00	01	02	03
B	00	10	20	30	01	11	21	31	02	12	22	32	03	13	23	33	10	11	12	13	10	11	12	13
C	00	10	20	30	01	11	21	31	02	12	22	32	03	13	23	33	20	21	22	23	20	21	22	23
D	00	10	20	30	01	11	21	31	02	12	22	32	03	13	23	33	30	31	32	33	30	31	32	33
E	00	10	20	30	01	11	21	31	02	12	22	32	03	13	23	33	00	01	02	03	00	01	02	03
F	00	10	20	30	01	11	21	31	02	12	22	32	03	13	23	33	10	11	12	13	10	11	12	13
G	00	10	20	30	01	11	21	31	02	12	22	32	03	13	23	33	20	21	22	23	20	21	22	23
H	00	10	20	30	01	11	21	31	02	12	22	32	03	13	23	33	30	31	32	33	30	31	32	33
I	00	10	20	30	01	11	21	31	02	12	22	32	03	13	23	33	00	01	02	03				
J	00	10	20	30	01	11	21	31	02	12	22	32	03	13	23	33	10	11	12	13				
K	00	10	20	30	01	11	21	31	02	12	22	32	03	13	23	33	20	21	22	23				
L	00	10	20	30	01	11	21	31	02	12	22	32	03	13	23	33	30	31	32	33				
M	00	10	20	30	01	11	21	31	02	12	22	32	03	13	23	33	00	01	02	03				
N	00	10	20	30	01	11	21	31	02	12	22	32	03	13	23	33	10	11	12	13				
O	00	10	20	30	01	11	21	31	02	12	22	32	03	13	23	33	20	21	22	23				
P	00	10	20	30	01	11	21	31	02	12	22	32	03	13	23	33	30	31	32	33				

Chapter 5 treatment plate layout

The plate layout overleaf shows which treatment was placed in each well for the selection experiment described in Chapter 5. The key to the plate layout is as follows:

Green shading- antibiotic-free control treatment

Purple shading- blank wells with no bacterial culture. These blanks were used to monitor levels of background noise in the luminescence reading. These wells were transferred to fresh plates in the same way as the wells containing bacterial cultures, to detect if the transfer process was introducing contamination to cultures.

Orange shading- these wells were inoculated with saturated bacterial culture and used for calibrating the spectrometer only.

The first digit in each two digit-code for the treatments represents the frequency with which antibiotics were rotated. c indicates a constant antibiotic treatment, 1 a treatment where antibiotics were switched every day, 2 a treatment where antibiotics were switched every 2 days, and 4 a treatment where antibiotics were switched every 4 days.

The second digit represents the order in which antibiotics were applied. C indicates that ceftriaxone was applied first, and S that sulfamethoxazole was applied first.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A		cC	cC	cS	cS	1C	1C	1C	1S	1S	1S	2C	2C	2C	2S	2S	2S	4C	4C	4C	4S	4S	4S	
B		cC	cC	cS	cS	1C	1C	1C	1S	1S	1S	2C	2C	2C	2S	2S	2S	4C	4C	4C	4S	4S	4S	
C		cC	cC	cS	cS	1C	1C	1C	1S	1S	1S	2C	2C	2C	2S	2S	2S	4C	4C	4C	4S	4S	4S	
D		cC	cC	cS	cS	1C	1C	1C	1S	1S	1S	2C	2C	2C	2S	2S	2S	4C	4C	4C	4S	4S	4S	
E		cC	cC	cS	cS	1C	1C	1C	1S	1S	1S	2C	2C	2C	2S	2S	2S	4C	4C	4C	4S	4S	4S	
F		cC	cC	cS	cS	1C	1C	1C	1S	1S	1S	2C	2C	2C	2S	2S	2S	4C	4C	4C	4S	4S	4S	
G		cC	cC	cS	cS	1C	1C	1C	1S	1S	1S	2C	2C	2C	2S	2S	2S	4C	4C	4C	4S	4S	4S	
H		cC	cC	cS	cS	1C	1C	1C	1S	1S	1S	2C	2C	2C	2S	2S	2S	4C	4C	4C	4S	4S	4S	
I		cC	cC	cS	cS	1C	1C	1C	1S	1S	1S	2C	2C	2C	2S	2S	2S	4C	4C	4C	4S	4S	4S	
J		cC	cC	cS	cS	1C	1C	1C	1S	1S	1S	2C	2C	2C	2S	2S	2S	4C	4C	4C	4S	4S	4S	
K		cC	cC	cS	cS	1C	1C	1C	1S	1S	1S	2C	2C	2C	2S	2S	2S	4C	4C	4C	4S	4S	4S	
L		cC	cC	cS	cS	1C	1C	1C	1S	1S	1S	2C	2C	2C	2S	2S	2S	4C	4C	4C	4S	4S	4S	
M		cC	cC	cS	cS	1C	1C	1C	1S	1S	1S	2C	2C	2C	2S	2S	2S	4C	4C	4C	4S	4S	4S	
N		cC	cC	cS	cS	1C	1C	1C	1S	1S	1S	2C	2C	2C	2S	2S	2S	4C	4C	4C	4S	4S	4S	
O		cC	cC	cS	cS	1C	1C	1C	1S	1S	1S	2C	2C	2C	2S	2S	2S	4C	4C	4C	4S	4S	4S	
P		cC	cC	cS	cS	1C	1C	1C	1S	1S	1S	2C	2C	2C	2S	2S	2S	4C	4C	4C	4S	4S	4S	

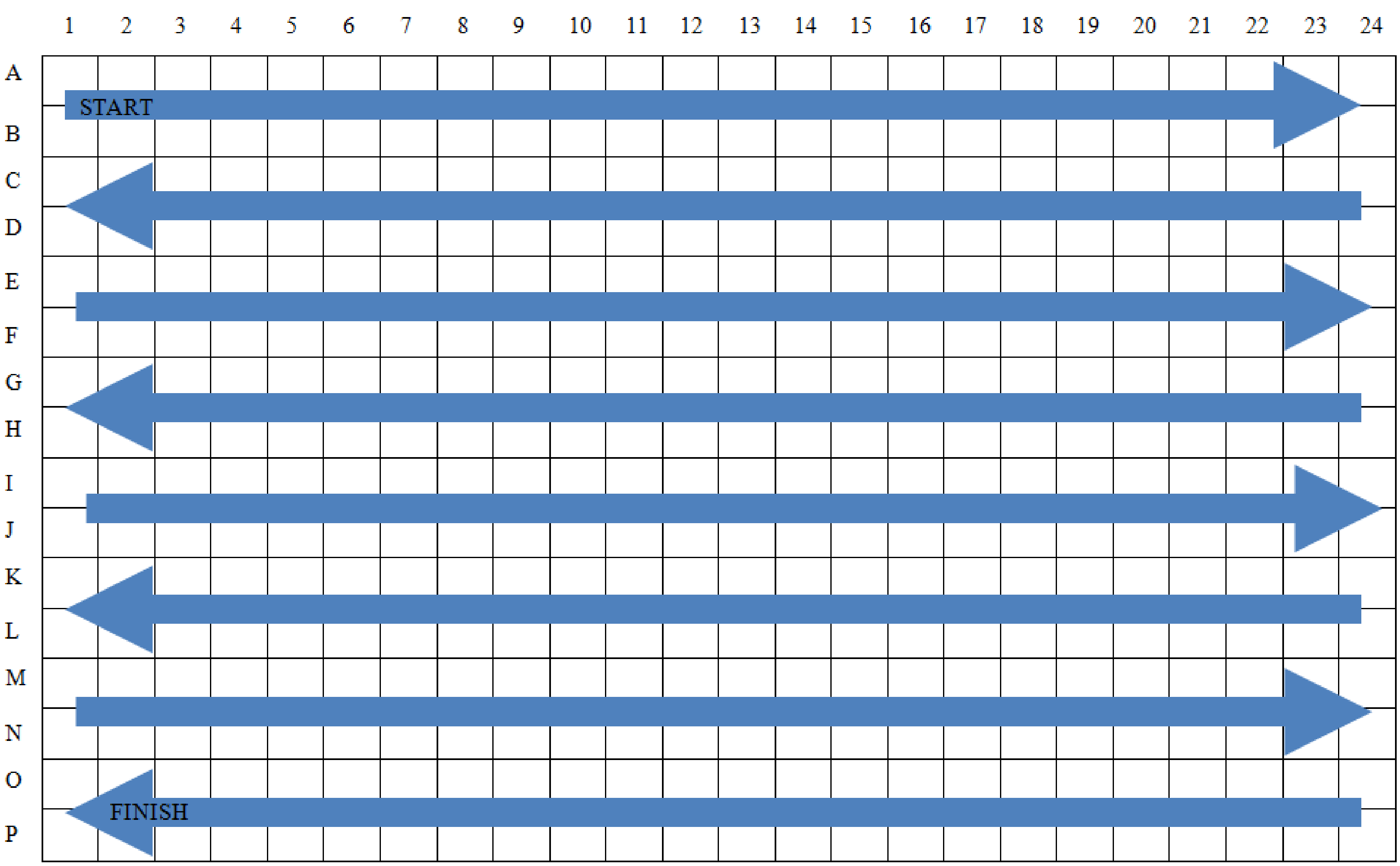
Direction in which transfers were carried out between plates

All transfers of bacterial cultures between 384 well plates were carried out using an automated pipettor (Precision XS, BioTek) throughout this thesis. The 8 channel multi-channel pipettor function was used to transfer 8 cultures per pipetting action, moving in a horizontal direction across the plate as illustrated below (numbers in diagram overleaf represent the order in which the multi-channel pipettor moved across the plate). The total time to transfer a full 384 well plate using the automated pipettor was approximately 10 minutes. Wells 24O and 24P were not transferred as these wells were used only for calibrating the spectrometer. In order to improve the accuracy of dilution, dilution was carried out through multiple transfers to new plates diluting by 10 fold each time.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	41	43	45	47
B	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48
C	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	41	43	45	47
D	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48
E	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	41	43	45	47
F	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48
G	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	41	43	45	47
H	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48
I	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	41	43	45	47
J	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48
K	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	41	43	45	47
L	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48
M	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	41	43	45	47
N	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48
O	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	41	43	45	
P	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	

Direction in which the spectrometer read the luminescence of wells within 384-well plates

The spectrometer used in the experiments reported in this thesis (FLUOStar OPTIMA, BMG Labtech) read horizontally back and forth across the 384-well plates, gradually moving down the plate, as illustrated in the diagram overleaf.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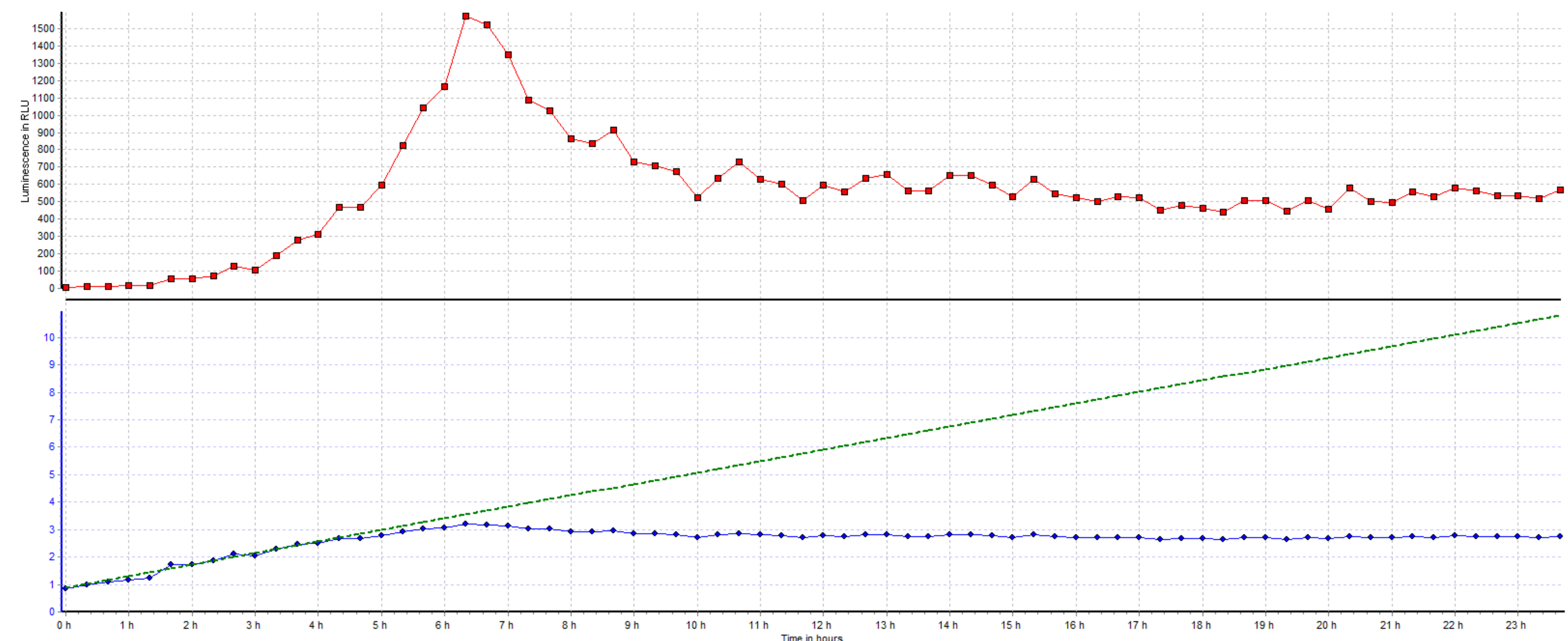
20
21
22
23
24
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
START
FINISH

Appendix C: Example curve showing the calculation of exponential growth rate

An example of a growth rate curve for an individual bacterial population is shown overleaf. On the upper graph (red points and trend line), relative luminescence units are plotted against time in hours. Relative luminescent units are a measure of the luminescence of a population, relative to the luminescence of a sample well measured immediately before the start of the 24 hour growth period. The lower graph (blue points and trend line) shows $\log_{10}$ relative luminescence units against time in hours. The green line shows the steepest gradient on the $\log_{10}$ luminescence- time graph over a five hour window: this is the exponential growth rate.

The first two phases of bacterial growth are lag phase and log phase. Lag phase is the period after bacteria are inoculated into a new culture medium before there is a substantial increase in cell numbers. During this phase cells increase in size but do not divide. Following lag phase, a bacterial population enters log phase, during which cells divide and the population size increases in a logarithmic fashion. The whole time period up to peak luminescence was counted as log phase for the purposes of calculating exponential growth rate.

Blanking was not carried out as the amount of noise in control wells containing media only did not exceed 20 relative luminescence units, which was a very small proportion of the luminescence of wells containing bacterial populations.


Luminescence in RLU
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
10
9
8
7
6
5
4
3
2
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8 h
9 h
10 h
11 h
12 h
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16 h
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21 h
22 h
23 h
Time in hours

Appendix D: ANOVA tables and diagnostic plots for all general linear models in the thesis

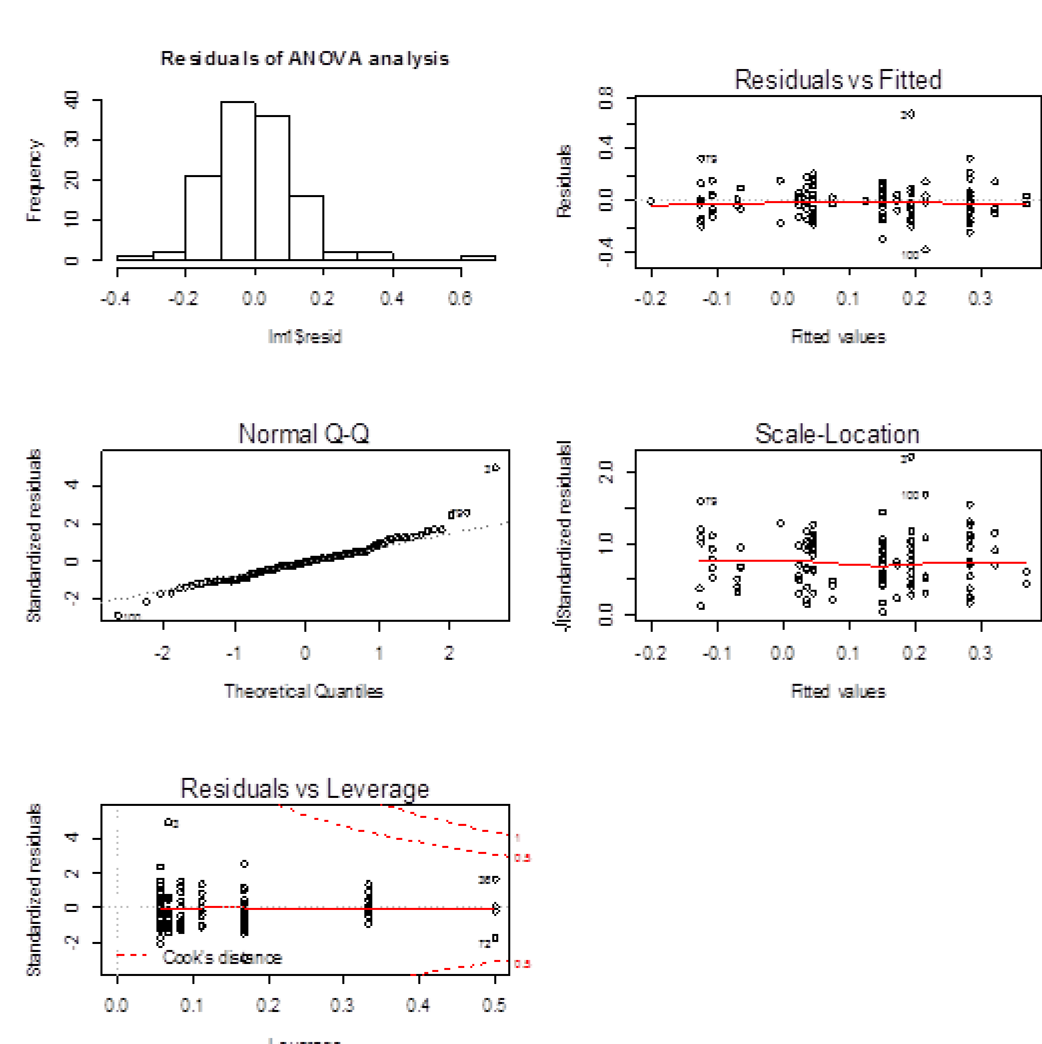
ANOVA tables and diagnostic plots begin on next page.

Chapter 2

GLM 1: S = block

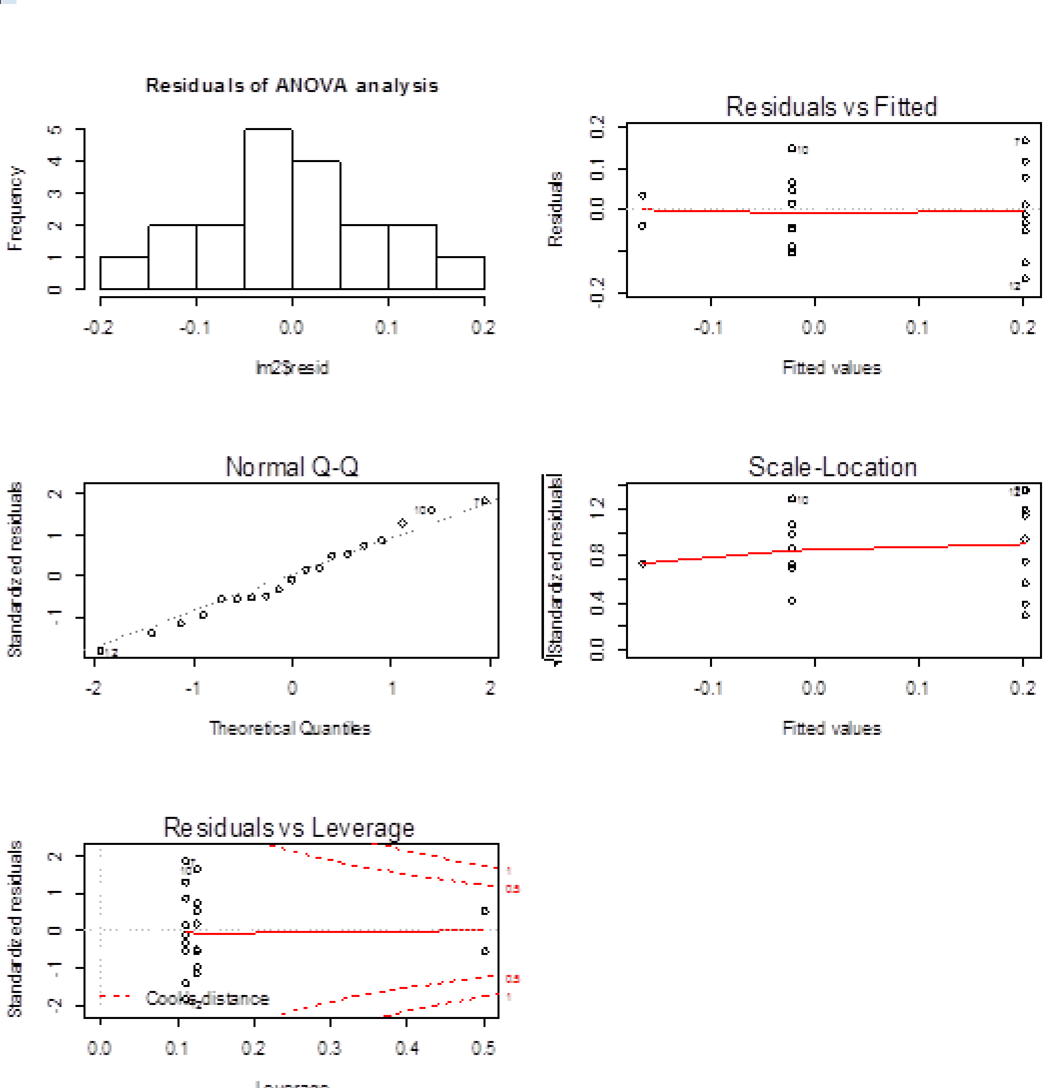
We found that block (i.e. a term coding the two antibiotic families that are interacting, as indicated in Fig. 2.1 / Table 2.2) had an effect on the degree of synergy ($F_{18, 101}$ = 6.30, P < 0.001).

```
Analysis of Variance Table

Response: S
           Df Sum Sq  Mean Sq F value     Pr(>F)
block      18 2.2739 0.126325  6.2997 4.534e-10 ***
Residuals 101 2.0253 0.020053
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```



GLM 2: S = Code representing whether bactericidal or bacteriostatic antibiotics were included in the treatment

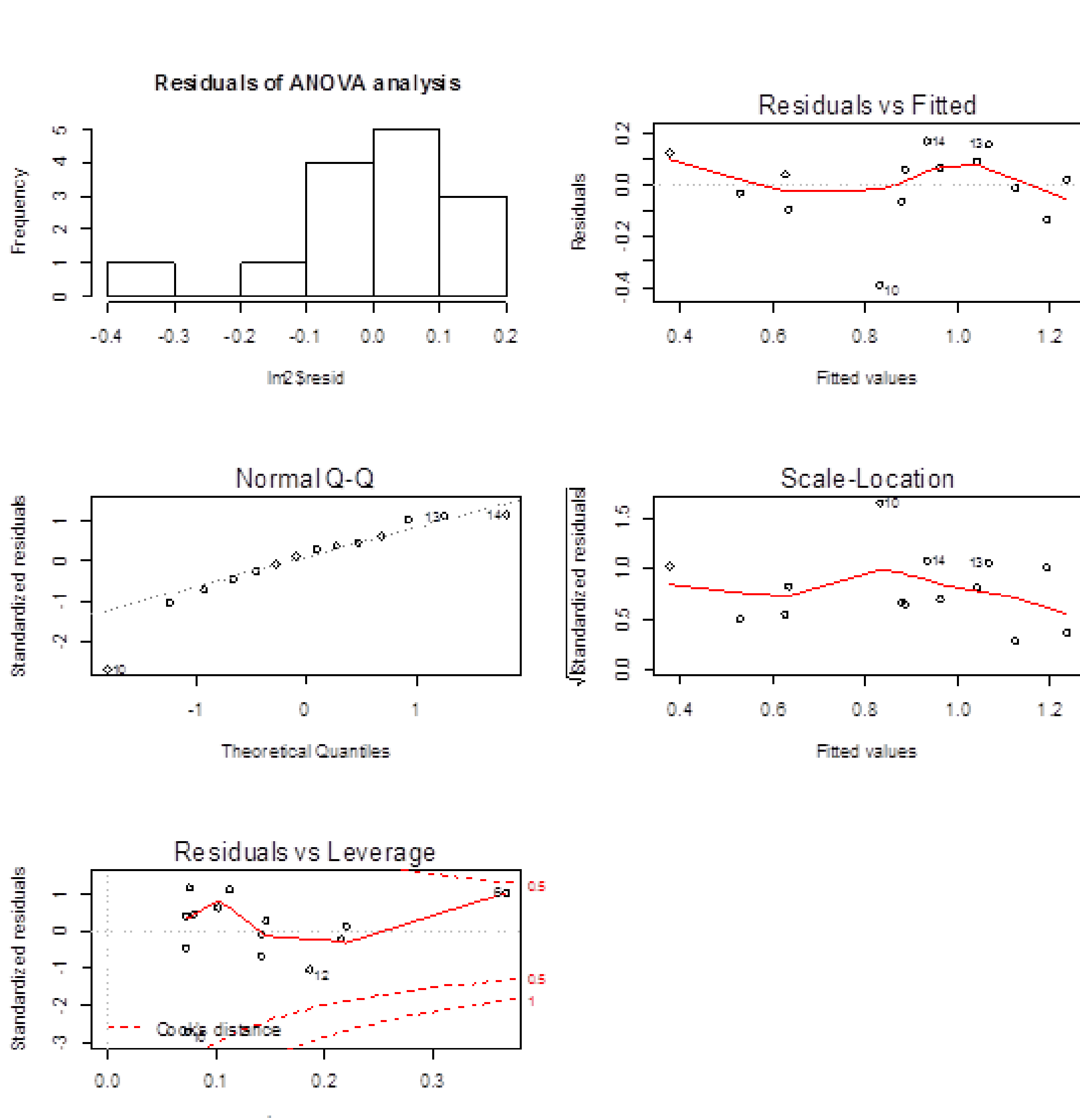
```
> anova(lm2)
Analysis of Variance Table

Response: S
                 Df  Sum Sq Mean Sq F value    Pr(>F)
Cidal_static_code  2 0.33562 0.16781  17.928 8.214e-05 ***
Residuals        16 0.14976 0.00936
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
>
```



Chapter 3

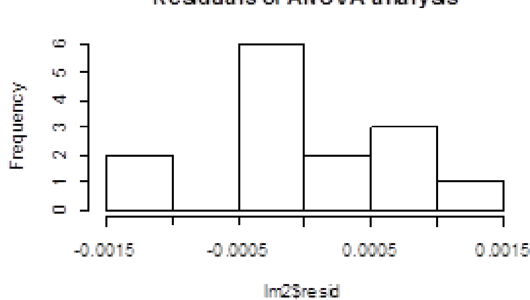
GLM 1: Growth rate of ancestral strain = degree of synergy

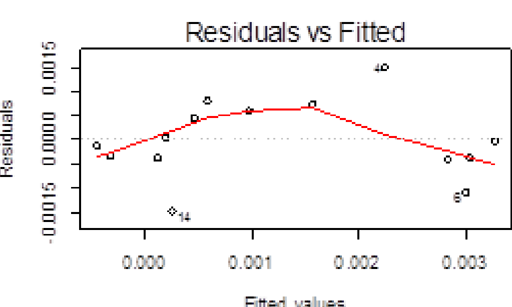
```
> anova(lm2)
Analysis of Variance Table

Response: Ancestor..growth.rate
                  Df  Sum Sq Mean Sq F value    Pr(>F)    
Degree.of.synergy  1 0.85652 0.85652  37.881 4.911e-05 ***
Residuals         12 0.27133 0.02261                      
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> 
```

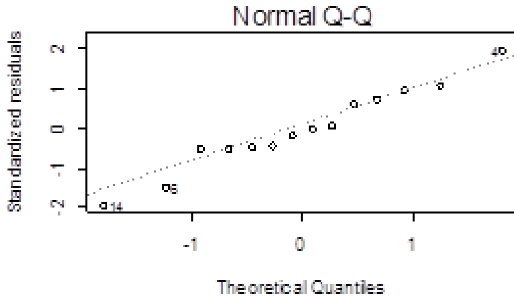


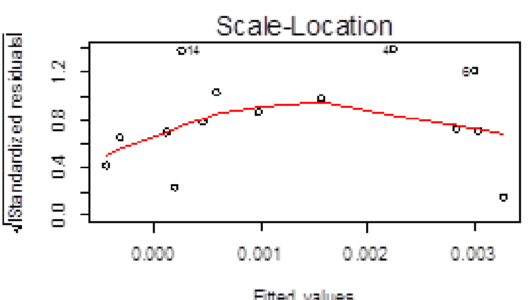
GLM 2: Rate of adaptation = growth rate of ancestral strain

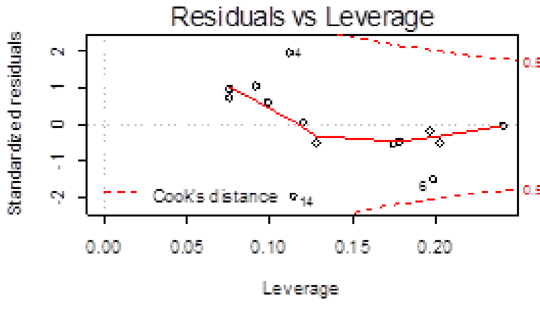
```
> anova(lm2)
Analysis of Variance Table

Response: Rate.of.adaptation
                     Df     Sum Sq    Mean Sq F value    Pr(>F)    
Ancestor..growth.rate  1 2.4056e-05 2.4056e-05  36.831 5.595e-05 ***
Residuals             12 7.8378e-06 6.5320e-07                      
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> 
```





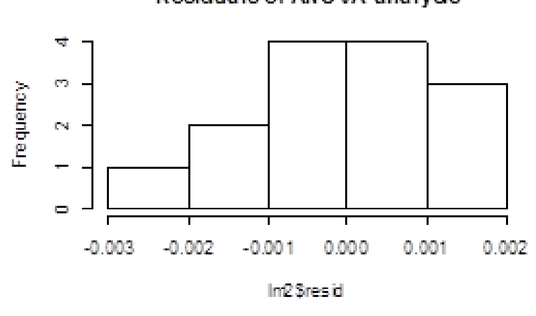


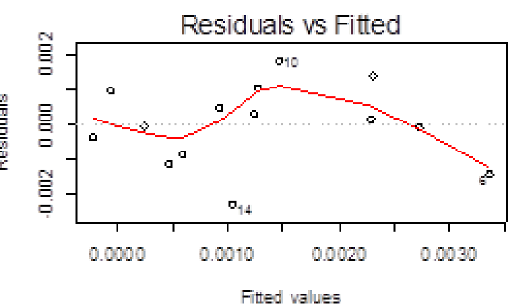


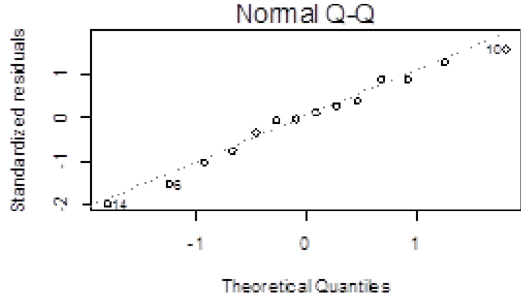


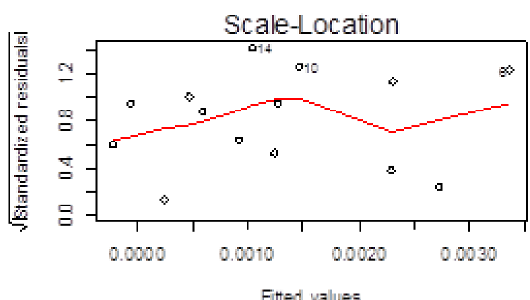
GLM 3: Rate of adaptation = degree of synergy

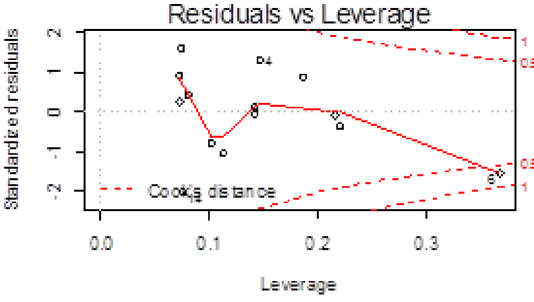
```
Analysis of Variance Table

Response: Rate.of.adaptation
                   Df     Sum Sq    Mean Sq F value   Pr(>F)
Degree.of.synergy   1 1.4960e-05 1.4960e-05  10.601 0.00688 **
Residuals          12 1.6934e-05 1.4111e-06
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> 
```





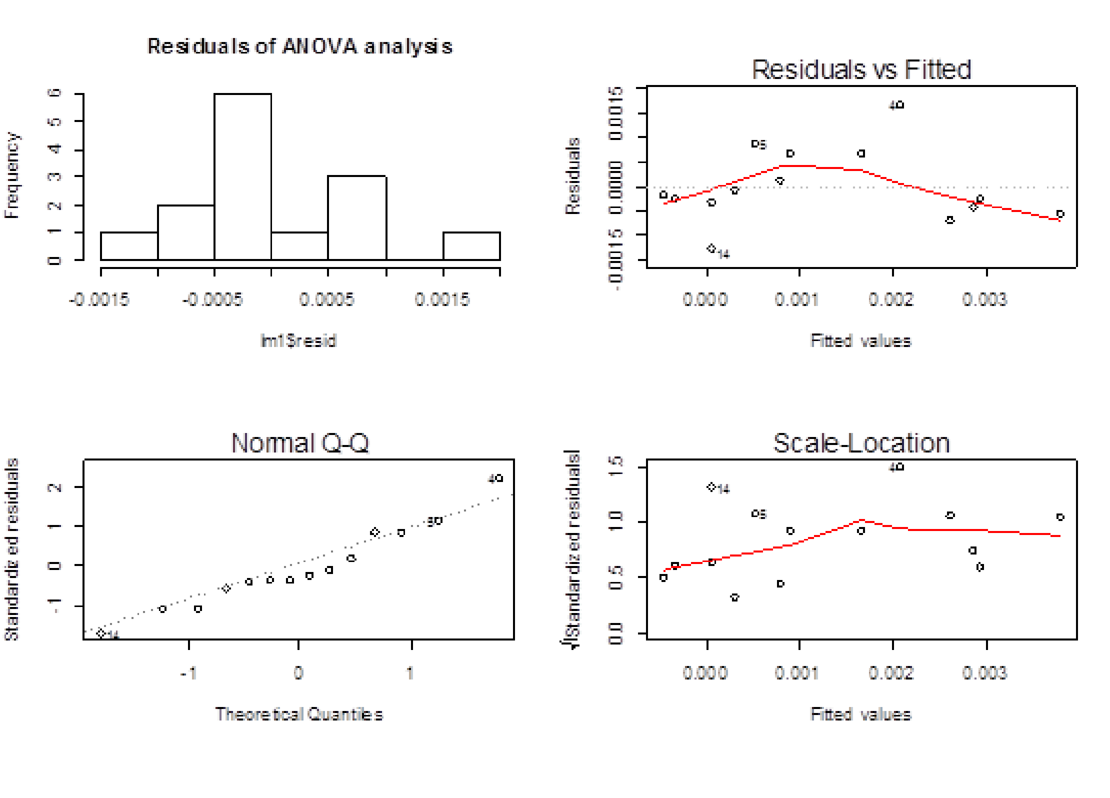


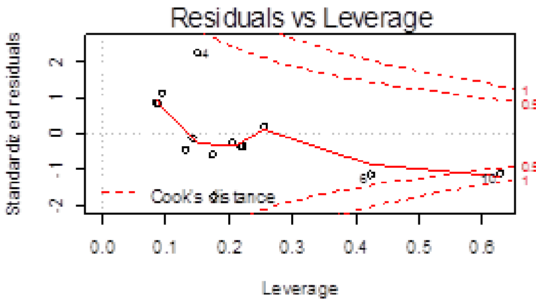




GLM 4: Rate of adaptation = Growth rate of ancestral strain + Degree of synergy

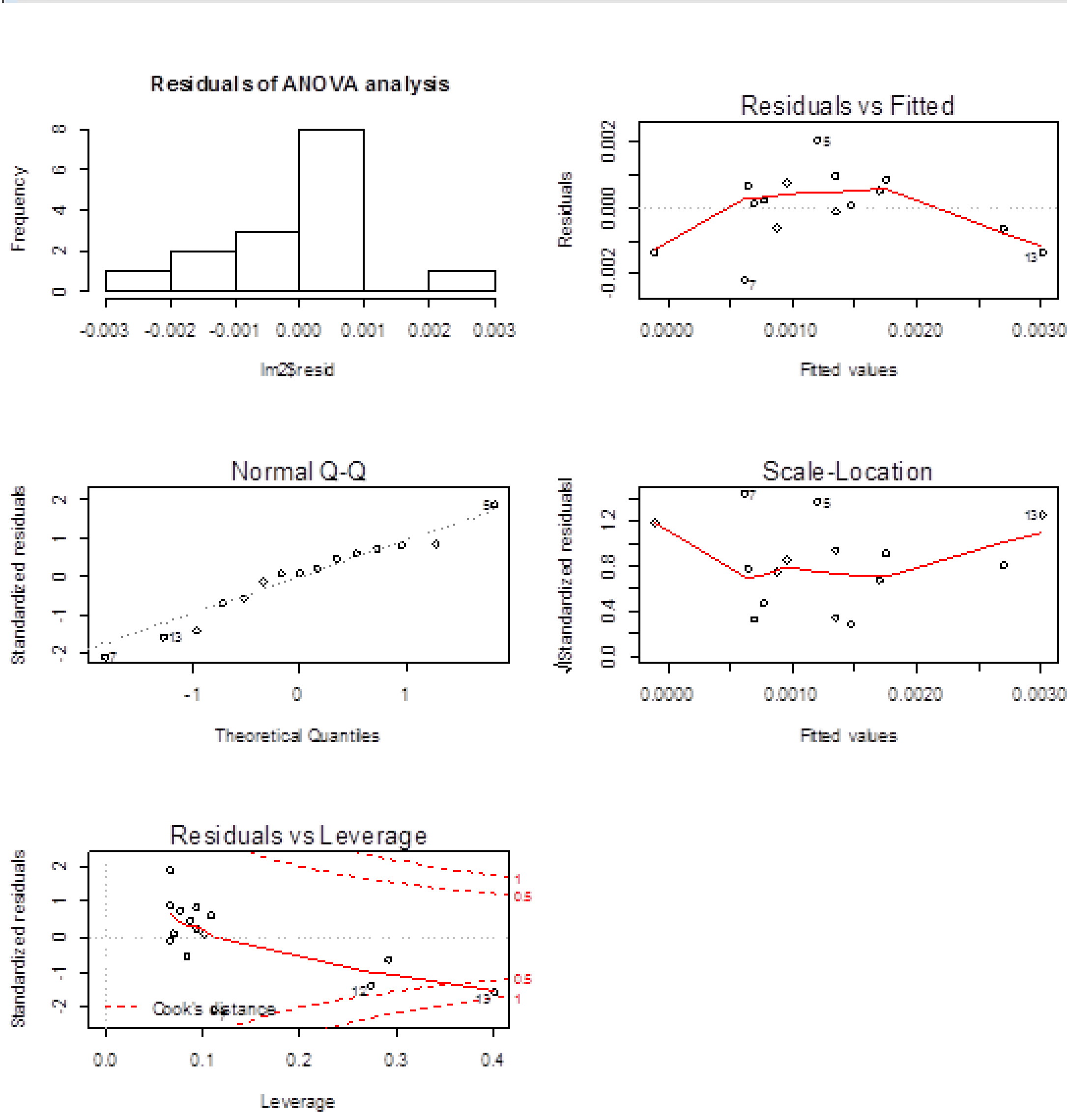
```
> anova(lm1)
Analysis of Variance Table

Response: Rate.of.adaptation
                      Df     Sum Sq    Mean Sq F value    Pr(>F)    
Ancestor..growth.rate  1 2.4056e-05 2.4056e-05 37.0023 7.927e-05 ***
Degree.of.synergy      1 6.8640e-07 6.8640e-07  1.0558    0.3262    
Residuals             11 7.1514e-06 6.5010e-07                      
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> 
```





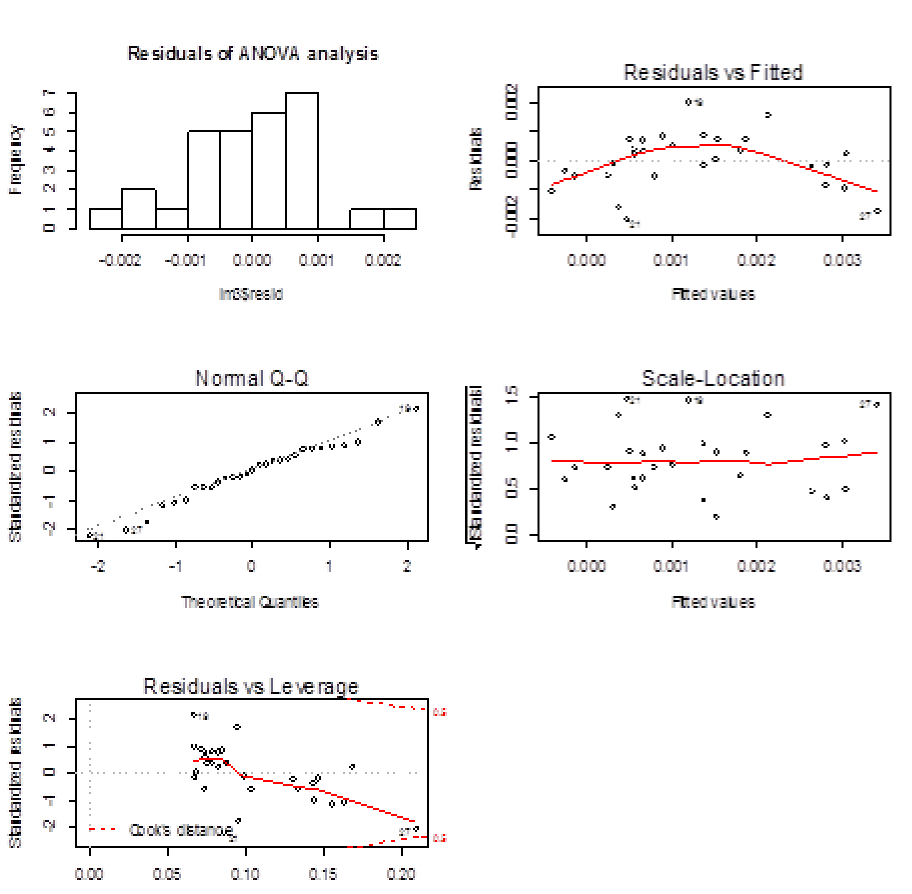
GLM 5: For populations evolved with an individual antibiotic- Rate of adaptation = growth rate of ancestral strain

```
> anova(lm2)
Analysis of Variance Table

Response: Individual.adaptation
                    Df     Sum Sq    Mean Sq F value   Pr(>F)
Individual.ancestor  1 9.1889e-06 9.1889e-06  7.2847 0.01823 *
Residuals           13 1.6398e-05 1.2614e-06
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> |
```



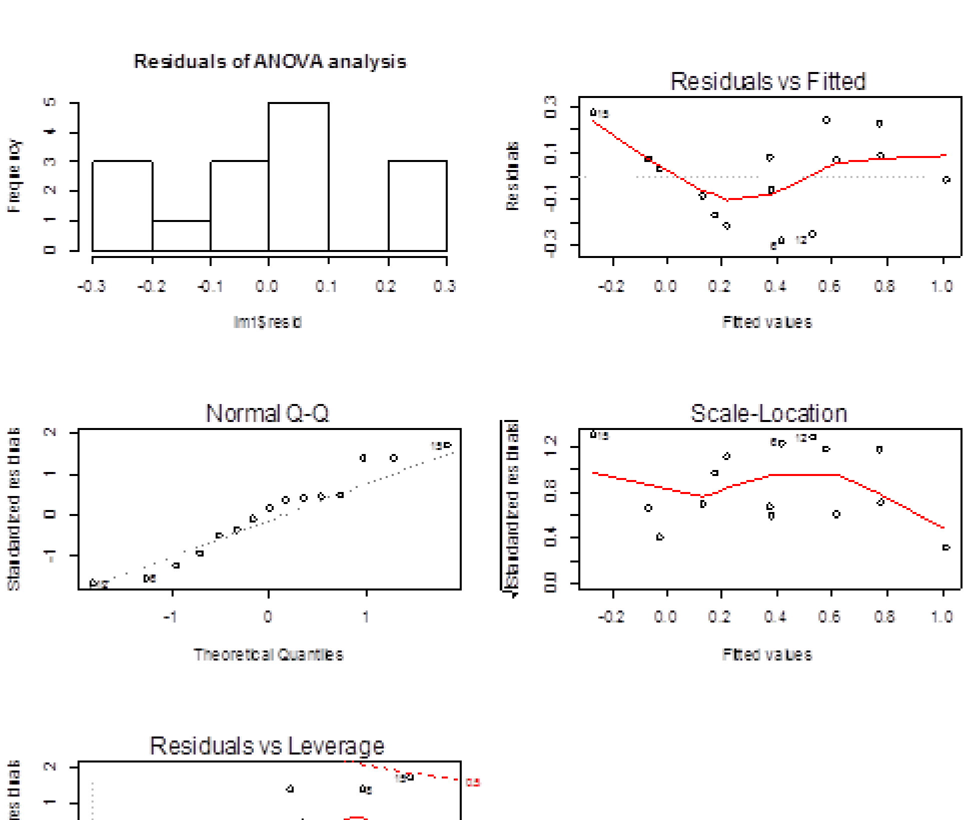
GLM 6: Rate of adaptation = growth rate of ancestral strain + code for whether treatment was an individual antibiotic or combination treatment

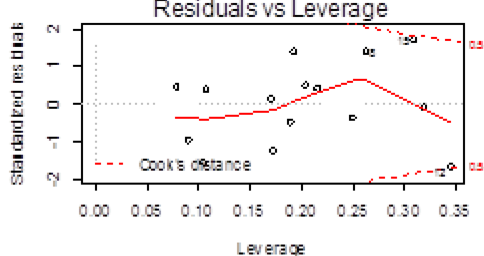
```
Call:
lm(formula = Rate.of.adaptation ~ Ancestor.growth.rate + Combination.or.indivi

Residuals:
       Min         1Q     Median         3Q        Max
-0.0020458 -0.0005108  0.0000428  0.0007357  0.0020439

Coefficients:
                                Estimate Std. Error t value Pr(>|t|)
(Intercept)                    0.0048358  0.0006696   7.222 1.14e-07 ***
Ancestor.growth.rate          -0.0040639  0.0007005  -5.802 4.11e-06 ***
Combination.or.individual[T.i] 0.0003058  0.0003684   0.830    0.414
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.0009816 on 26 degrees of freedom
  (1 observation deleted due to missingness)
Multiple R-squared: 0.5642,     Adjusted R-squared: 0.5307
F-statistic: 16.83 on 2 and 26 DF,  p-value: 2.045e-05
```



Chapter 4 GLM 1: Survival rate = concentration of ceftriaxone + concentration of sulfamethoxazole

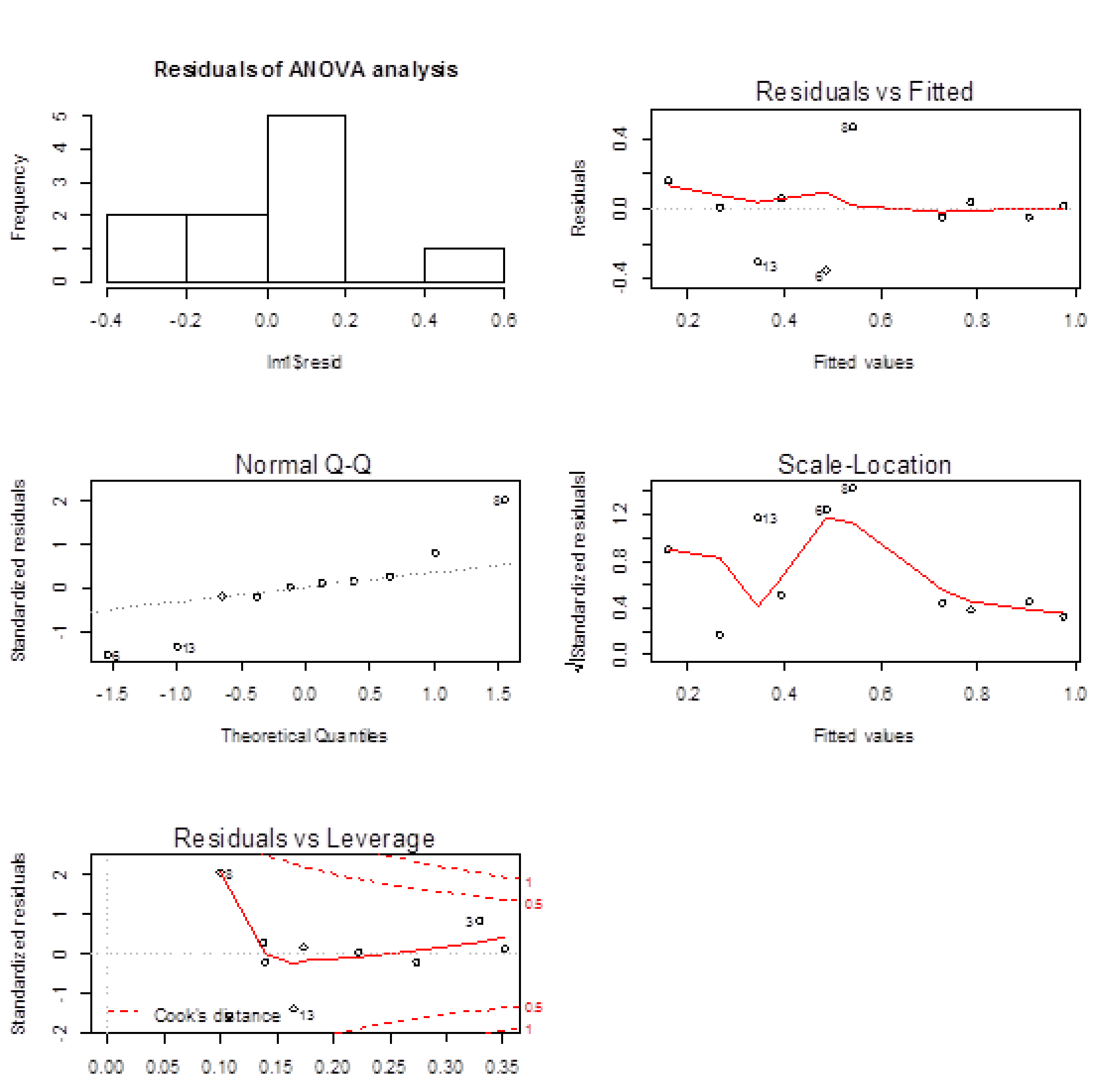
```
Call:
lm(formula = survival ~ conccefx + concsulfa)

Residuals:
     Min       1Q   Median       3Q      Max 
-0.27860 -0.12717  0.02846  0.08465  0.27186 

Coefficients:
              Estimate Std. Error t value Pr(>|t|)    
(Intercept)  1.1773442  0.1259057   9.351 7.36e-07 ***
conccefx    -0.0800148  0.0141939  -5.637 0.000109 ***
concsulfa   -0.0032453  0.0006721  -4.828 0.000413 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.191 on 12 degrees of freedom
Multiple R-squared: 0.8008,	Adjusted R-squared: 0.7676 
F-statistic: 24.13 on 2 and 12 DF,  p-value: 6.24e-05

> 
```





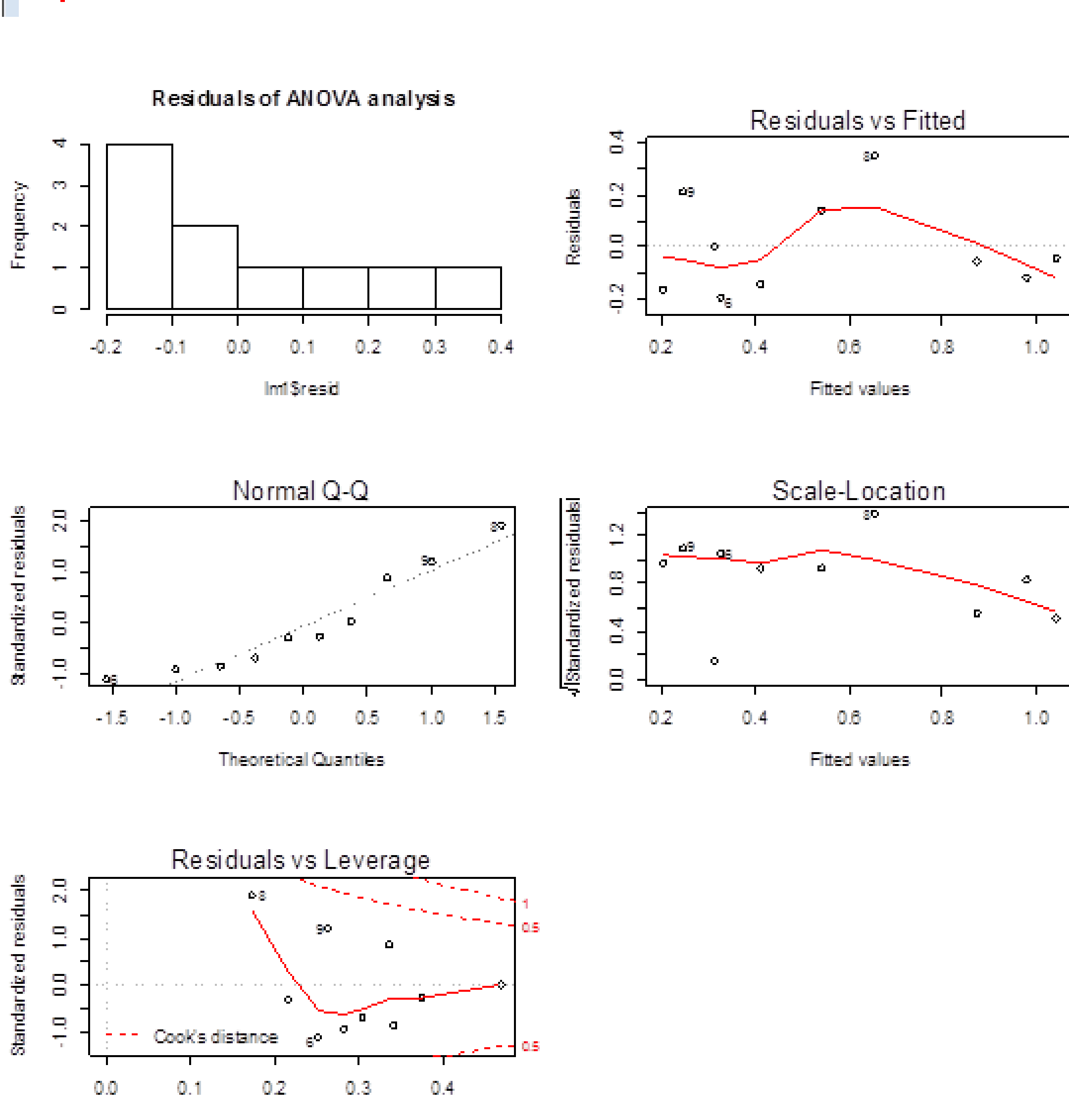
GLM 2: Survival rate = growth rate of ancestral strain

```
> anova(lm1)
Analysis of Variance Table

Response: survival
               Df  Sum Sq Mean Sq F value   Pr(>F)
initial.fitness  1 0.70001 0.70001  12.248 0.008083 **
Residuals        8 0.45723 0.05715
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> 
```



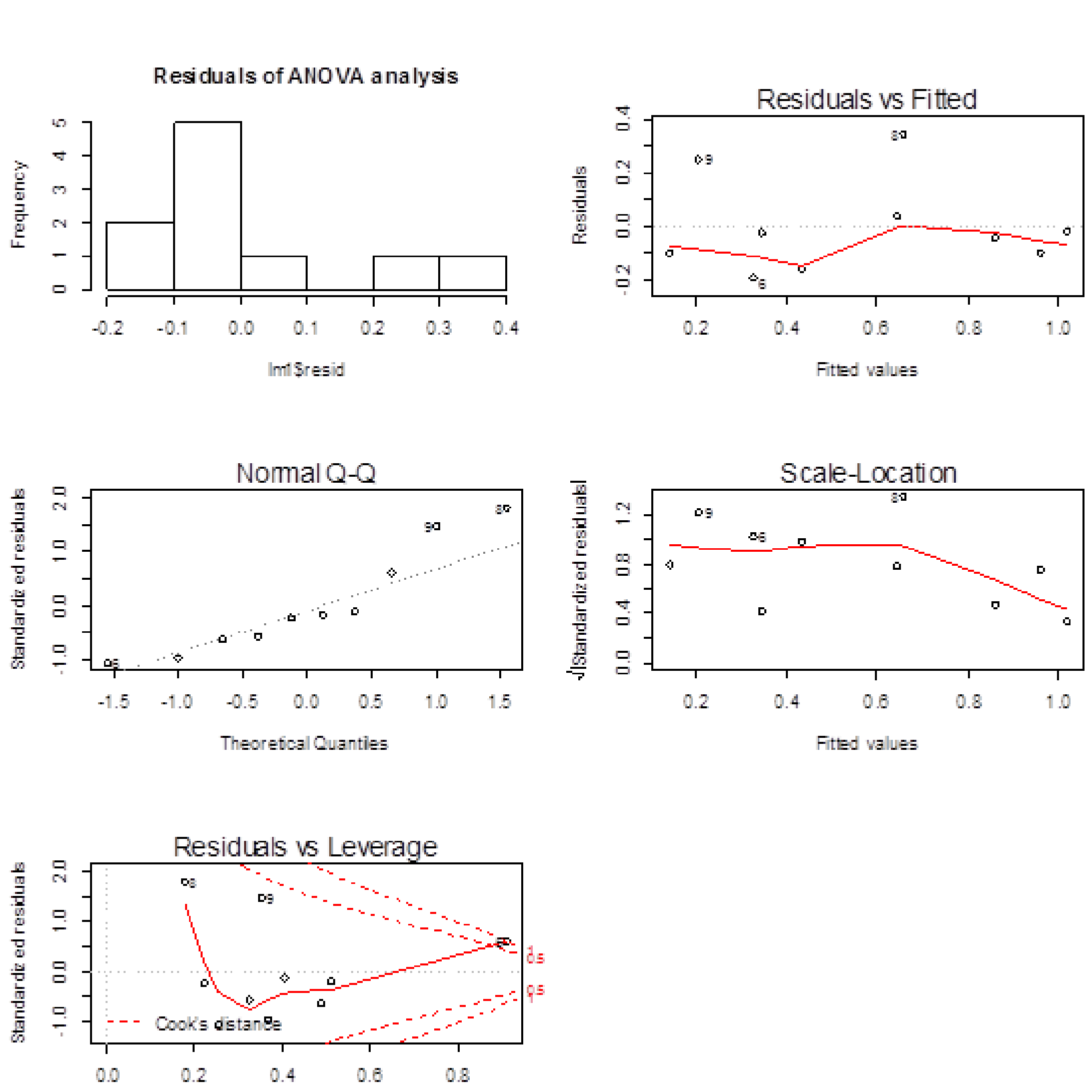
GLM 3: Survival rate = Growth rate of ancestral strain + Code for whether treatment was an individual antibiotic or combination treatment

```
Analysis of Variance Table

Response: survival
                 Df  Sum Sq Mean Sq F value   Pr(>F)
initial.fitness   1 0.70001 0.70001 17.4369 0.004159 **
combind           1 0.17621 0.17621  4.3893 0.074411 .
Residuals         7 0.28102 0.04015
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> |
```



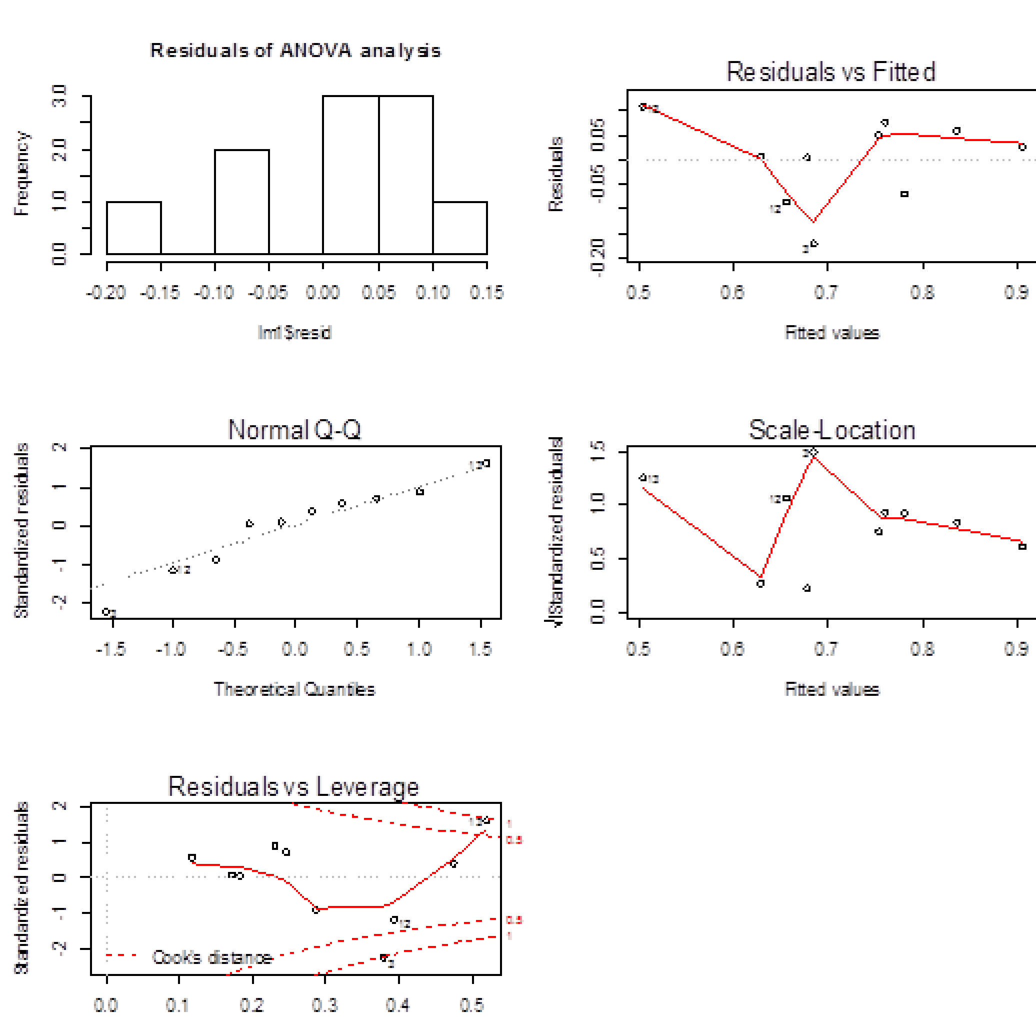
GLM 4: Survival rate = Growth rate of ancestral strain x Code for whether treatment was an individual antibiotic or combination treatment

```
Analysis of Variance Table

Response: survival
                        Df  Sum Sq Mean Sq F value   Pr(>F)
initial.fitness          1 0.70001 0.70001 15.9913 0.007128 **
combind                  1 0.17621 0.17621  4.0254 0.091618 .
initial.fitness:combind  1 0.01837 0.01837  0.4197 0.541086
Residuals                6 0.26265 0.04377
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
>
```



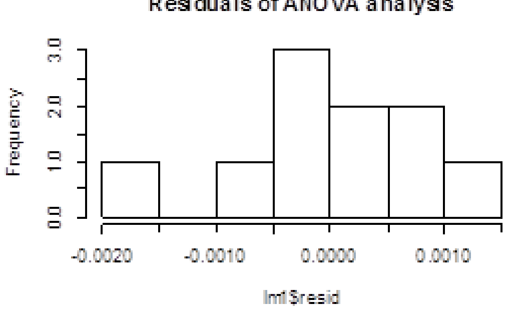
GLM 5: Growth rate of ancestral strain = concentration of ceftriaxone + concentration of sulfamethoxazole

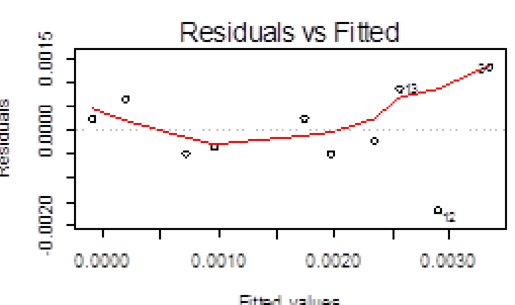
```
Call:
lm(formula = initial.fitness ~ conccefx + concsulfa)

Residuals:
     Min       1Q   Median       3Q      Max 
-0.17000 -0.05246  0.01666  0.05712  0.10627 

Coefficients:
              Estimate Std. Error t value Pr(>|t|)    
(Intercept)  0.9892703  0.0841782  11.752 7.31e-06 ***
conccefx    -0.0305227  0.0110027  -2.774   0.0275 *  
concsulfa   -0.0016690  0.0004932  -3.384   0.0117 *  
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.09637 on 7 degrees of freedom
  (5 observations deleted due to missingness)
Multiple R-squared: 0.6437,     Adjusted R-squared: 0.5419 
F-statistic: 6.323 on 2 and 7 DF,  p-value: 0.027
```

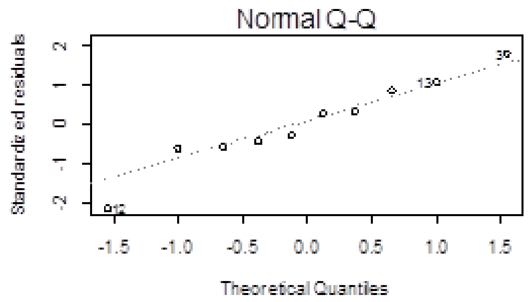


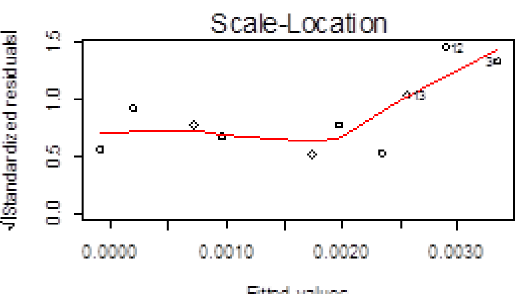
GLM 6: Rate of adaptation = growth rate of ancestral strain

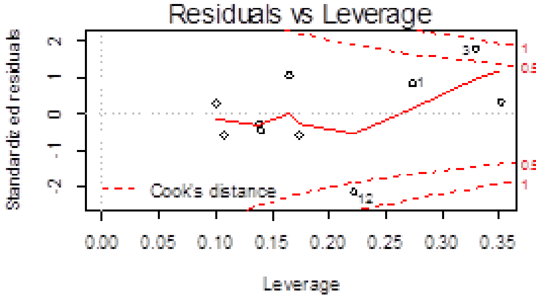
```
> anova(lm1)
Analysis of Variance Table

Response: slope
               Df     Sum Sq   Mean Sq F value    Pr(>F)
initial.fitness  1 1.2350e-05 1.235e-05  15.254 0.004509 **
Residuals        8 6.4773e-06 8.097e-07
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
>
```





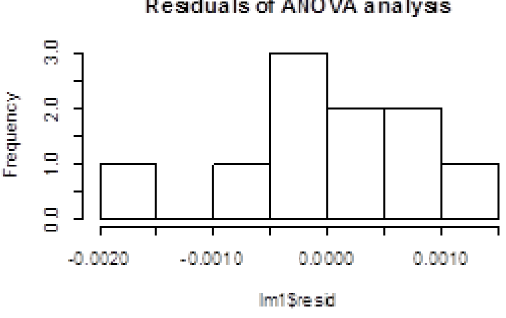


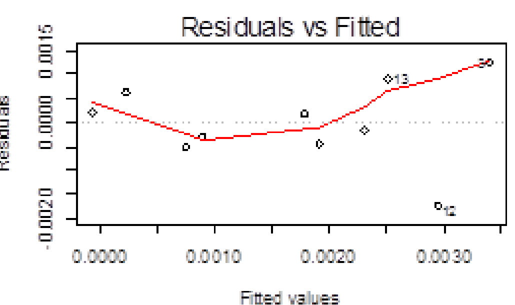


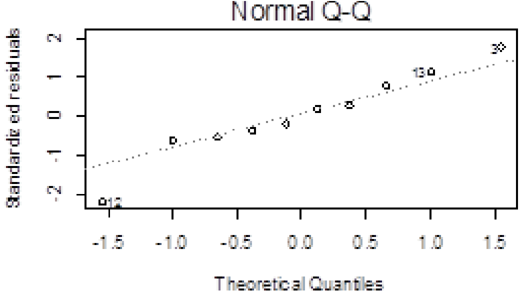


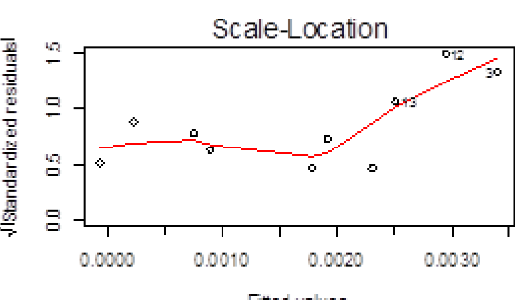
GLM 7: Rate of adaptation = Growth rate of ancestral strain + Code for whether treatment was an individual antibiotic or combination treatment

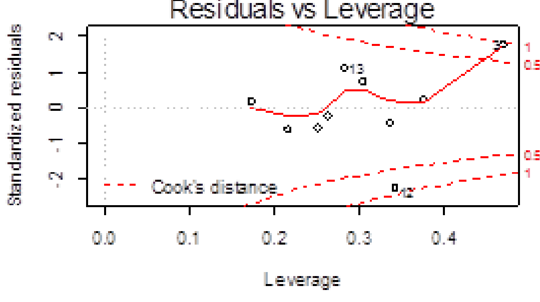
```
> anova(lm1)
Analysis of Variance Table

Response: slope
                Df     Sum Sq   Mean Sq F value    Pr(>F)
initial.fitness  1 1.2350e-05 1.235e-05 13.3923 0.008075 **
combind          1 2.1800e-08 2.180e-08  0.0236 0.882146
Residuals        7 6.4555e-06 9.222e-07
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
>
```





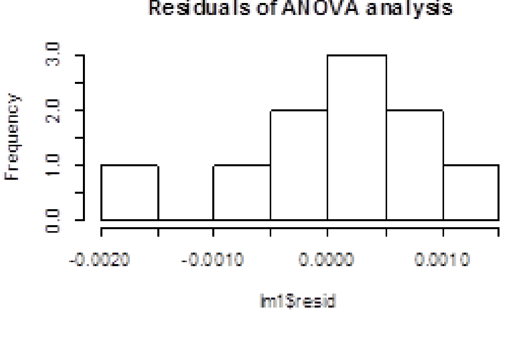


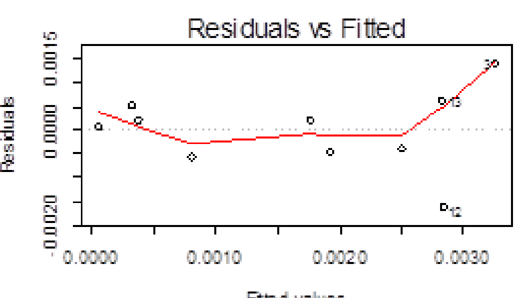


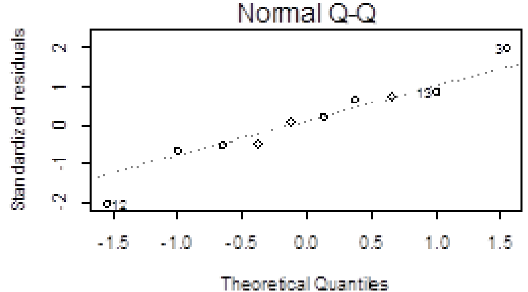


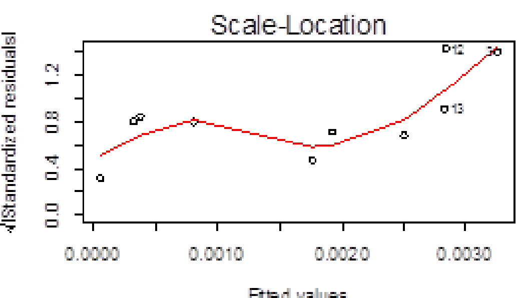
GLM 8: Rate of adaptation = Growth rate of ancestral strain x Code for whether treatment was an individual antibiotic or combination treatment

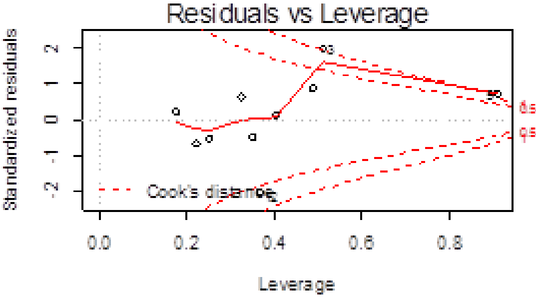
```
> anova(lm1)
Analysis of Variance Table

Response: slope
                        Df     Sum Sq   Mean Sq F value   Pr(>F)
initial.fitness          1 1.2350e-05 1.235e-05 12.3832 0.01253 *
combind                  1 2.1800e-08 2.180e-08  0.0219 0.88731
initial.fitness:combind  1 4.7130e-07 4.713e-07  0.4726 0.51748
Residuals                6 5.9841e-06 9.974e-07
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
>
```





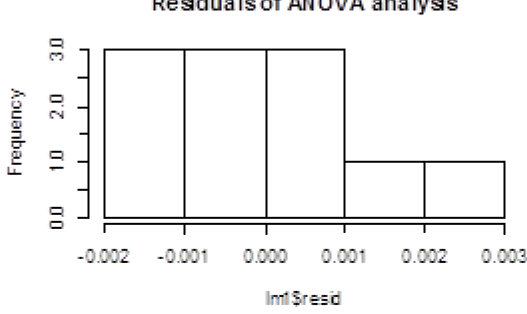


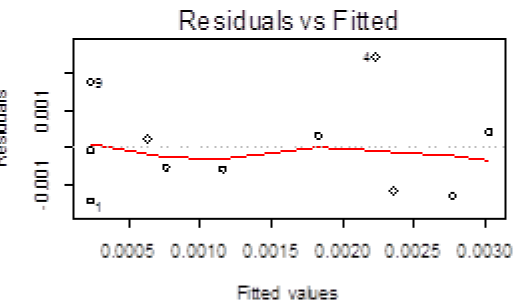


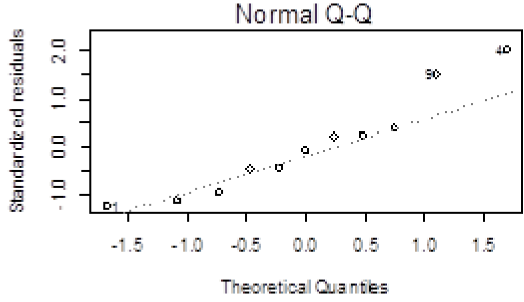


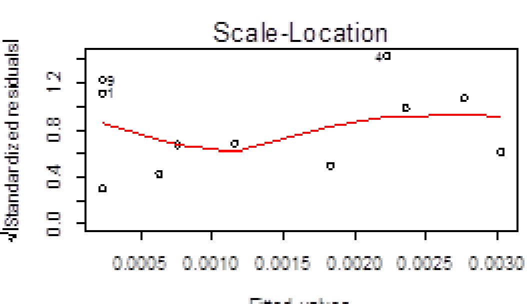
GLM 9: Rate of adaptation = Survival rate

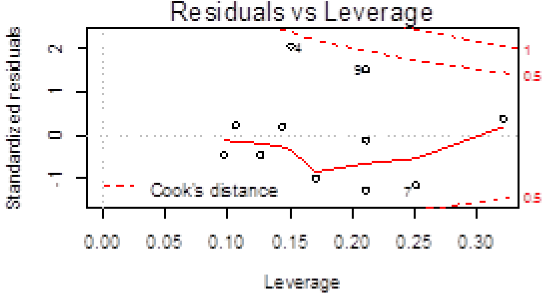
```
Analysis of Variance Table

Response: slope
          Df     Sum Sq    Mean Sq F value Pr(>F)
survival   1 1.1499e-05 1.1499e-05  6.9591  0.027 *
Residuals  9 1.4872e-05 1.6524e-06
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
>
```





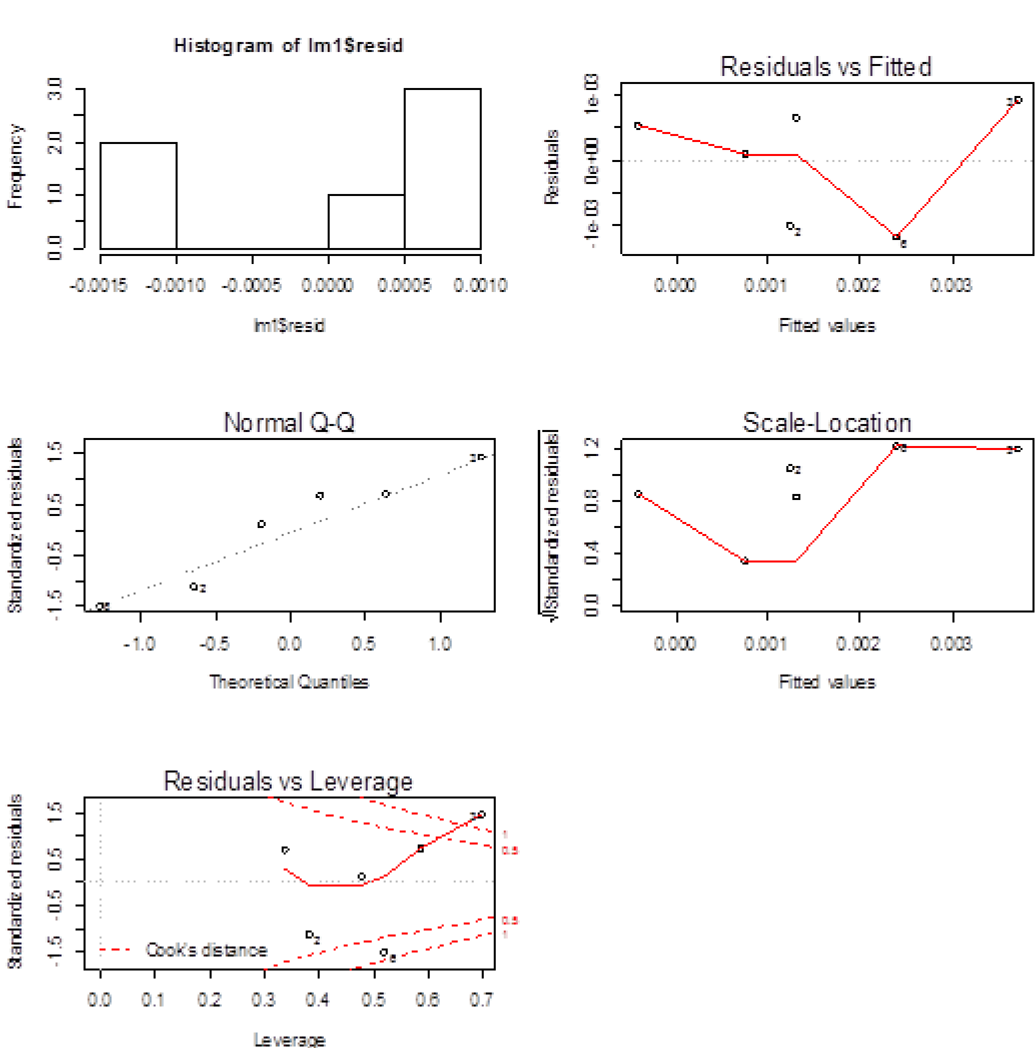






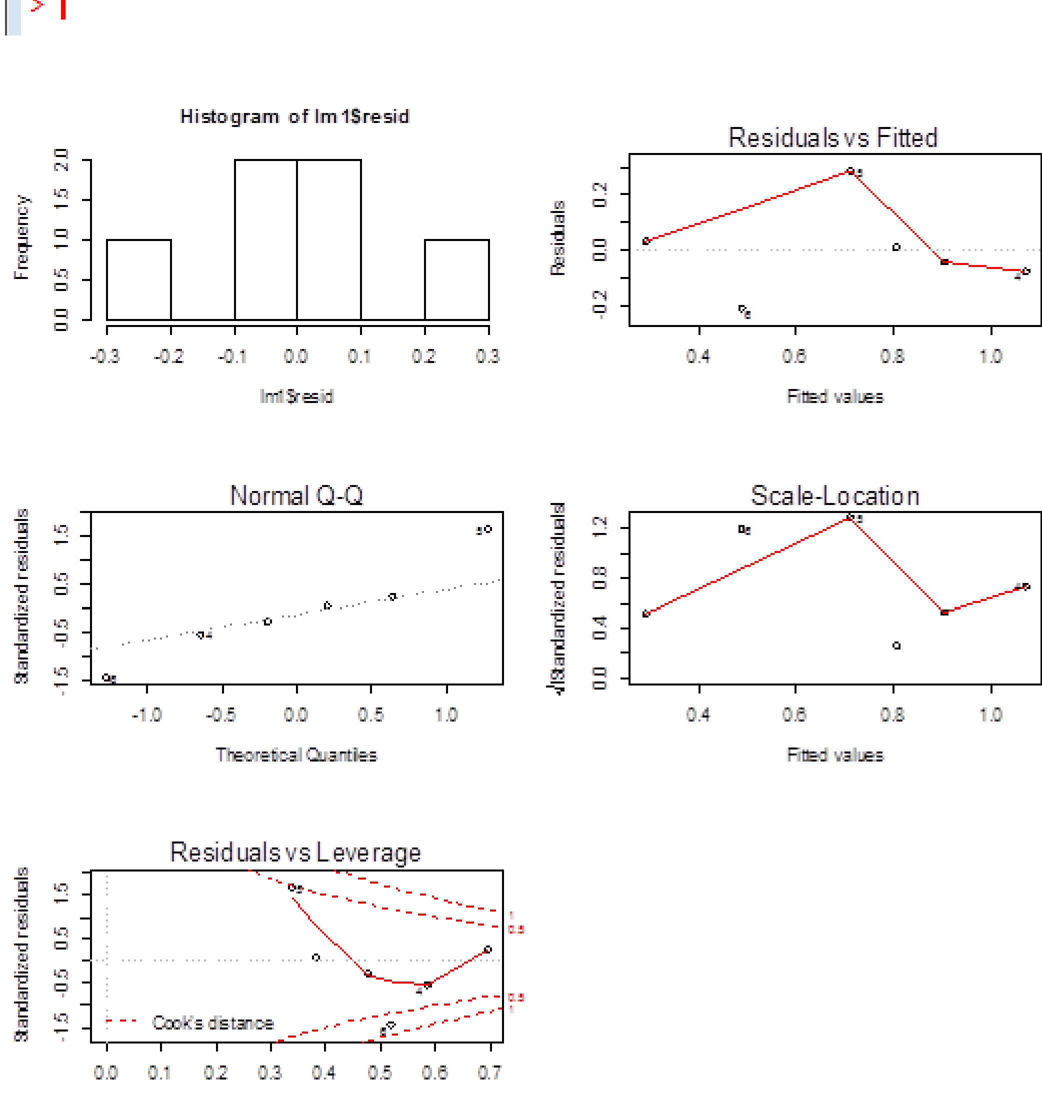
GLM 10: Rate of adaptation for individual treatments only = Growth rate of ancestral strain + Code for whether treatment was a ceftriaxone or sulfamethoxazole treatment

```
Analysis of Variance Table

Response: slopecefxsulfa
                  Df     Sum Sq    Mean Sq F value  Pr(>F)
initialcefxsulfa   1 8.9087e-06 8.9087e-06  6.6383 0.08203 .
cefxsulfa          1 1.1954e-06 1.1954e-06  0.8907 0.41491
Residuals          3 4.0261e-06 1.3420e-06
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
>
```

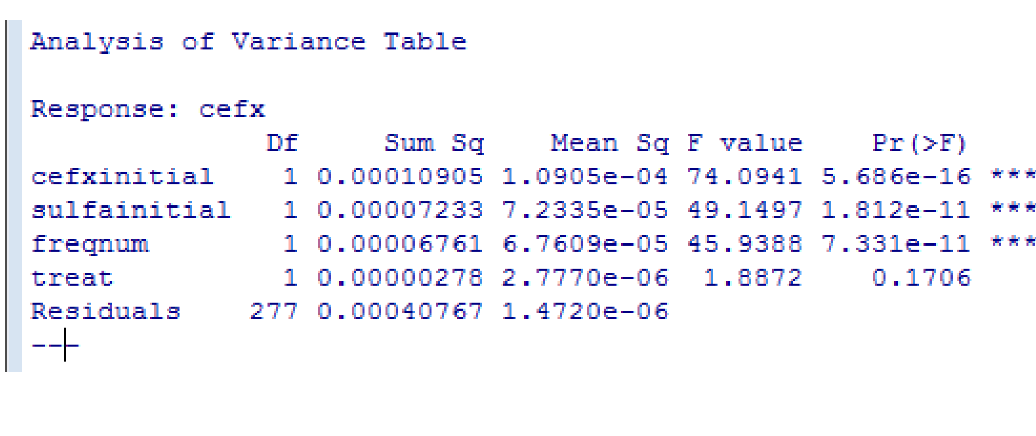


GLM 11: Survival rate for individual treatments only = Growth rate of ancestral strain + Code for whether treatment was a ceftriaxone or sulfamethoxazole treatment

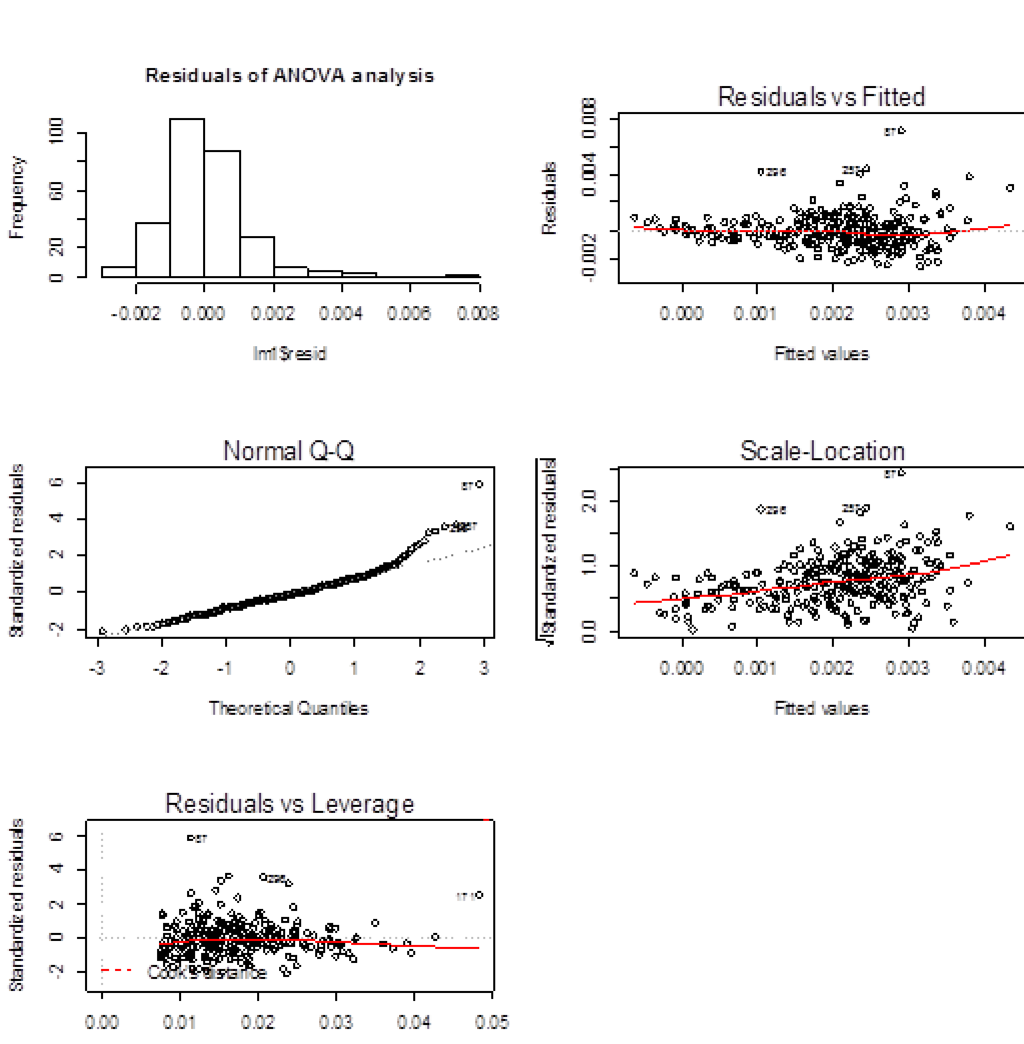
```
Analysis of Variance Table

Response: survivalcefxsulfa
                 Df  Sum Sq Mean Sq F value  Pr(>F)
initialcefxsulfa  1 0.39397 0.39397  8.6601 0.06037 .
cefxsulfa         1 0.01776 0.01776  0.3903 0.57643
Residuals         3 0.13648 0.04549
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
>
```



Chapter 5: GLM 1- Rate of adaptation to ceftriaxone = Growth rate of ancestral strain in ceftriaxone + Growth rate of ancestral strain in sulfamethoxazole + Frequency with which antibiotic environment changed (as a continuous variable) + Code for whether population started in a ceftriaxone or a sulfamethoxazole environment

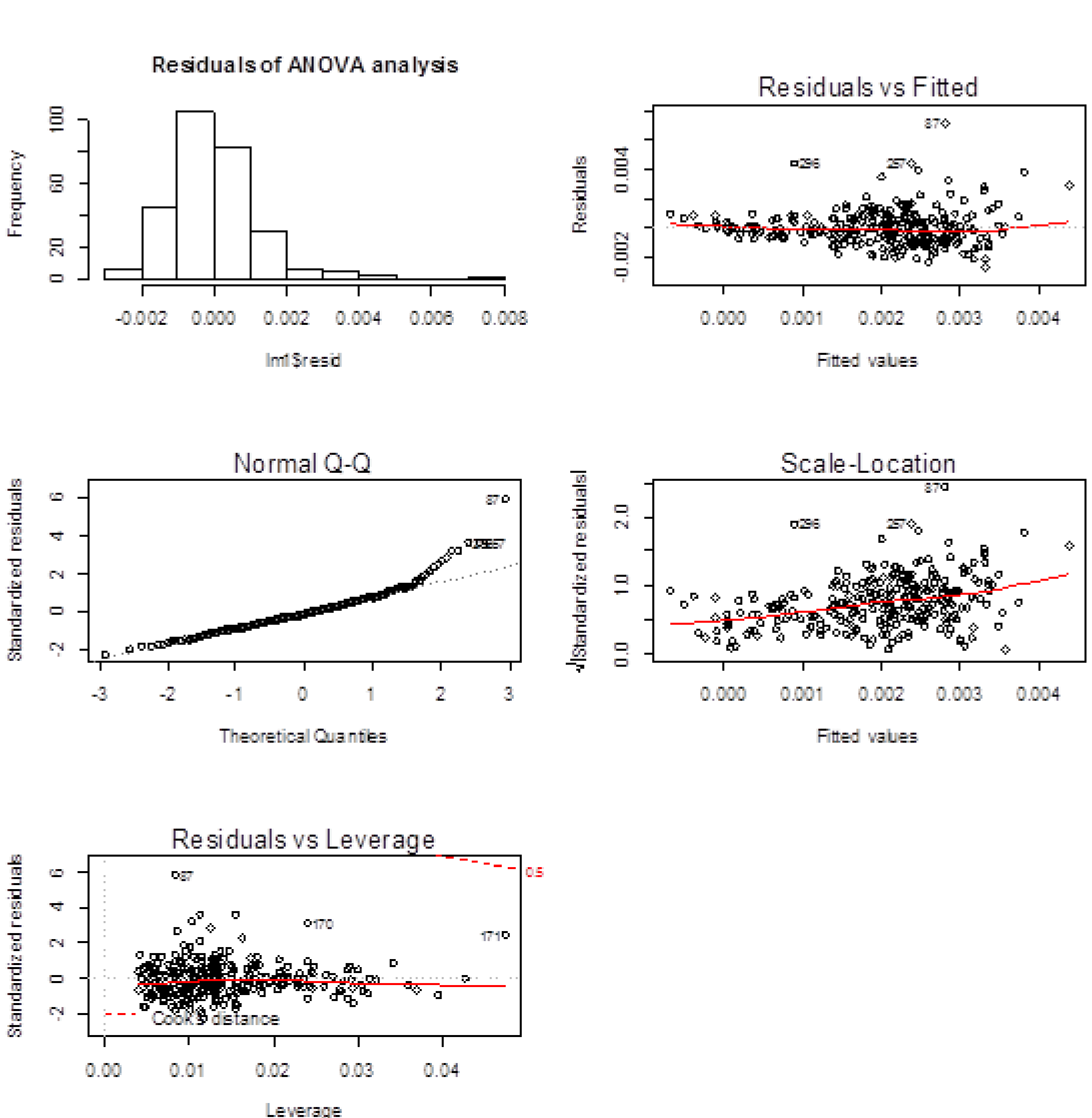


```
Analysis of Variance Table

Response: cefx
              Df     Sum Sq    Mean Sq F value    Pr(>F)
cefxinitial    1 0.00010905 1.0905e-04 74.0941 5.686e-16 ***
sulfainitial   1 0.00007233 7.2335e-05 49.1497 1.812e-11 ***
freqnum        1 0.00006761 6.7609e-05 45.9388 7.331e-11 ***
treat          1 0.00000278 2.7770e-06  1.8872    0.1706
Residuals    277 0.00040767 1.4720e-06
```



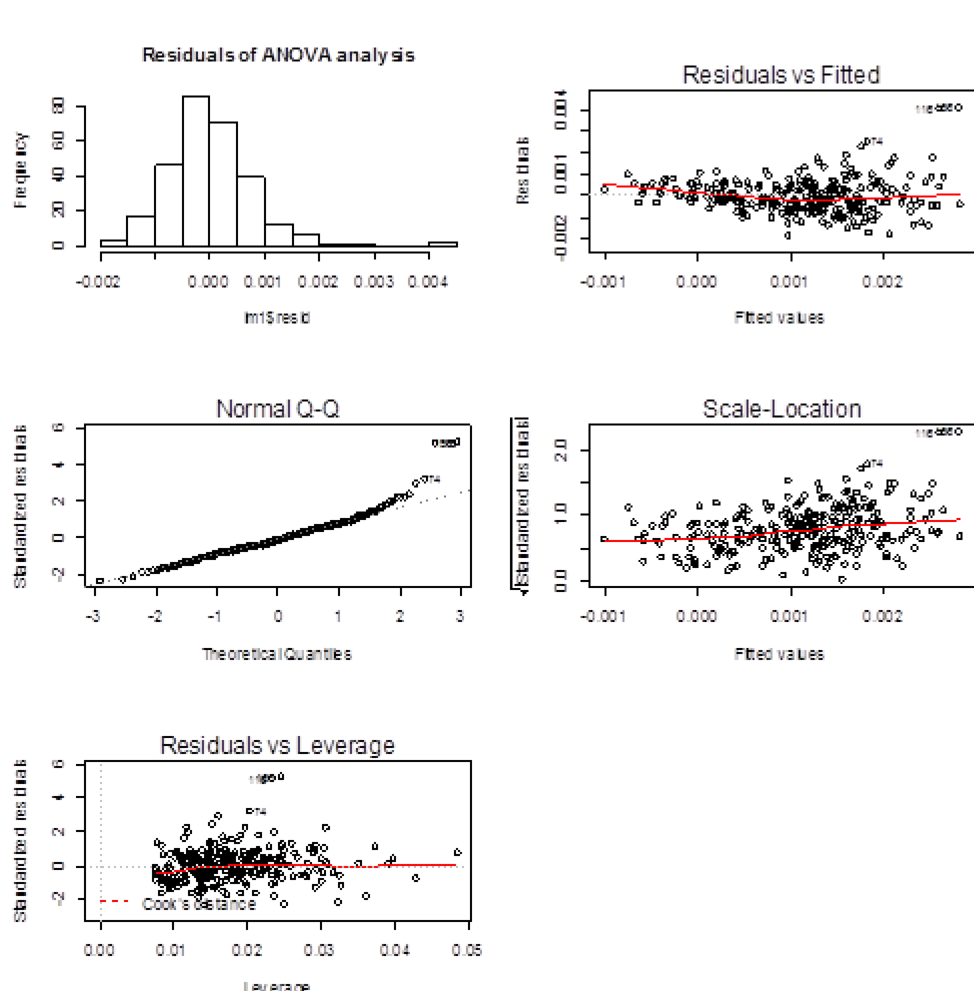
Simplified model with the insignificant Code for whether population started in a ceftriaxone or a sulfamethoxazole environment removed

```
Analysis of Variance Table

Response: cefx
              Df     Sum Sq    Mean Sq F value    Pr(>F)
cefxinitial    1 0.00010905 1.0905e-04  73.858 6.165e-16 ***
sulfainitial   1 0.00007233 7.2335e-05  48.993 1.926e-11 ***
freqnum        1 0.00006761 6.7609e-05  45.793 7.770e-11 ***
Residuals    278 0.00041045 1.4760e-06
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
>
```



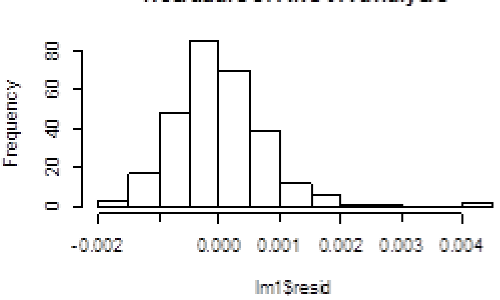
GLM 2 Rate of adaptation to sulfamethoxazole = Growth rate of ancestral strain in ceftriaxone + Growth rate of ancestral strain in sulfamethoxazole + Frequency with which antibiotic environment changed (as a continuous variable) + Code for whether population started in a ceftriaxone or a sulfamethoxazole environment

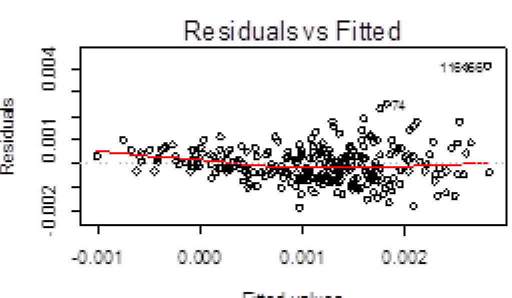
```
> anova(lm1)
Analysis of Variance Table

Response: sulfa
              Df     Sum Sq    Mean Sq  F value    Pr(>F)
cefxinitial    1 4.6131e-05 4.6131e-05  73.7108 6.627e-16 ***
sulfainitial   1 7.1273e-05 7.1273e-05 113.8843 < 2.2e-16 ***
freqnum        1 5.2192e-05 5.2192e-05  83.3959 < 2.2e-16 ***
treat          1 1.1000e-08 1.1000e-08   0.0172    0.8958
Residuals    277 1.7336e-04 6.2600e-07
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
>
```

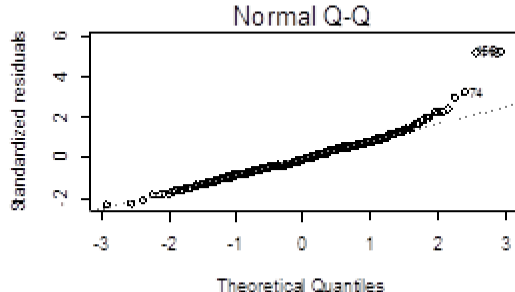


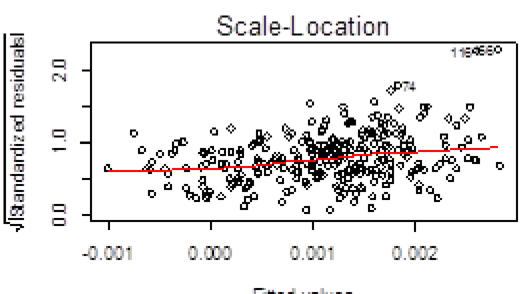
Simplified model with the insignificant Code for whether population started in a ceftriaxone or a sulfamethoxazole environment removed

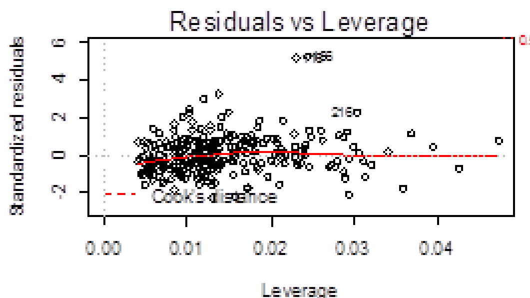
```
Analysis of Variance Table

Response: sulfa
              Df     Sum Sq    Mean Sq F value    Pr(>F)    
cefxinitial    1 4.6131e-05 4.6131e-05  73.972  5.89e-16 ***
sulfainitial   1 7.1273e-05 7.1273e-05 114.288 < 2.2e-16 ***
freqnum        1 5.2192e-05 5.2192e-05  83.692 < 2.2e-16 ***
Residuals    278 1.7337e-04 6.2400e-07                      
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> 
```



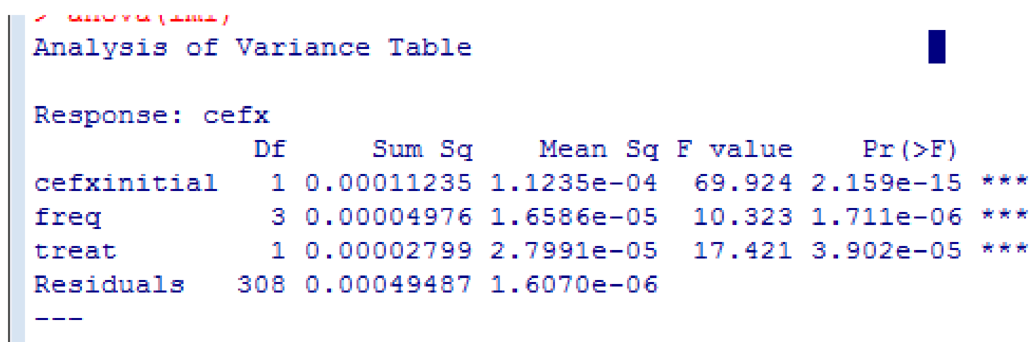




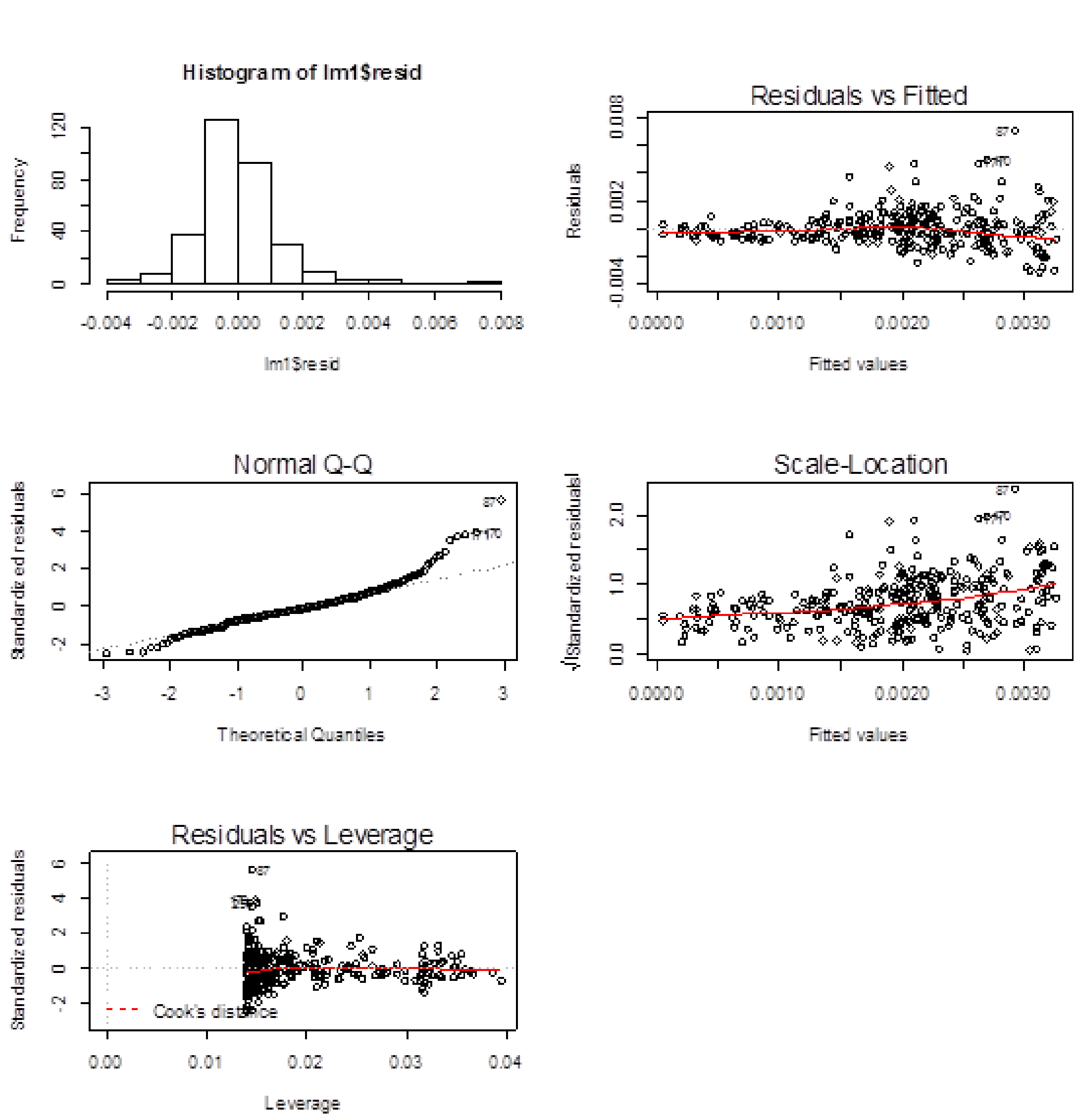




GLM 3: Rate of adaptation to ceftriaxone = Growth rate of ancestral strain in ceftriaxone + Frequency with which antibiotic environment changed (as a categorical variable) + Code for whether population started in a ceftriaxone or a sulfamethoxazole environment

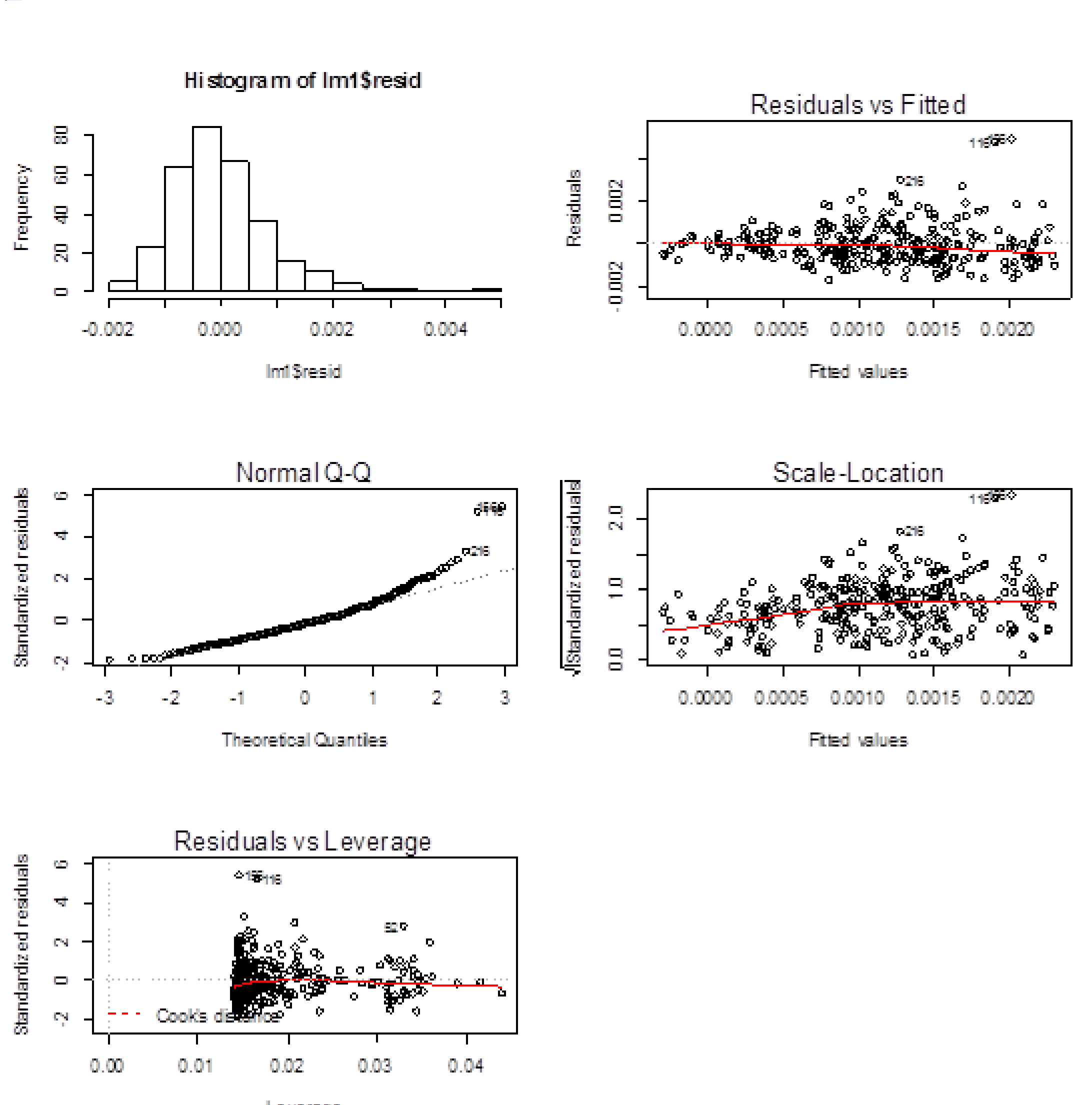


```
Analysis of Variance Table

Response: cefx
             Df     Sum Sq    Mean Sq F value    Pr(>F)
cefxinitial   1 0.00011235 1.1235e-04  69.924 2.159e-15 ***
freq          3 0.00004976 1.6586e-05  10.323 1.711e-06 ***
treat         1 0.00002799 2.7991e-05  17.421 3.902e-05 ***
Residuals   308 0.00049487 1.6070e-06
---
```



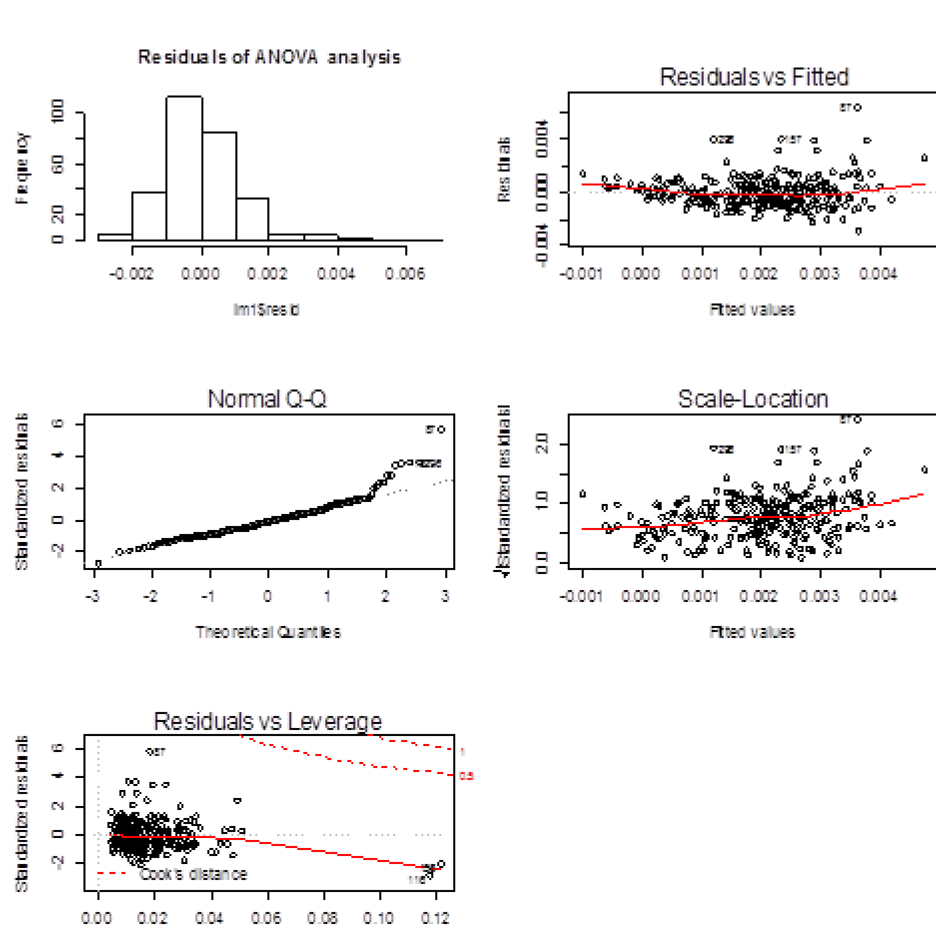
GLM 4: Rate of adaptation to sulfamethoxazole = Growth rate of ancestral strain in sulfamethoxazole + Frequency with which antibiotic environment changed (as a categorical variable) + Code for whether population started in a ceftriaxone or a sulfamethoxazole environment

```
Analysis of Variance Table

Response: sulfa
              Df     Sum Sq    Mean Sq F value    Pr(>F)
sulfainitial   1 3.7101e-05 3.7101e-05  44.657 1.100e-10 ***
freq           3 5.1723e-05 1.7241e-05  20.752 2.845e-12 ***
treat          1 2.1797e-05 2.1797e-05  26.236 5.337e-07 ***
Residuals    308 2.5589e-04 8.3100e-07
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
>
```



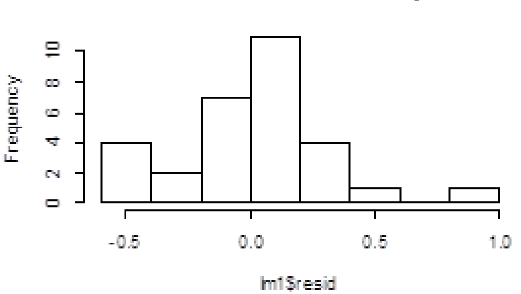
GLM 5: Rate of adaptation to ceftriaxone = Rate of adaptation to sulfamethoxazole + Growth rate of ancestral strain in ceftriaxone + Growth rate of ancestral strain in sulfamethoxazole + Frequency with which antibiotic environment changed

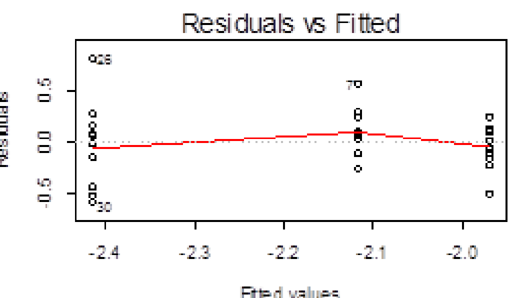
```
Call:
lm(formula = sulfa ~ cefx + sulfainitial + cefxinitial + freqnum,
    type = "II")

Residuals:
       Min         1Q     Median         3Q        Max
-0.0018243 -0.0004044 -0.0001000  0.0003784  0.0041475

Coefficients:
               Estimate Std. Error t value Pr(>|t|)
(Intercept)   1.429e-03  2.535e-04   5.637 4.27e-08 ***
cefx          2.592e-01  3.581e-02   7.237 4.53e-12 ***
sulfainitial -4.374e-03  3.588e-04 -12.190  < 2e-16 ***
cefxinitial   3.787e-03  2.614e-04  14.490  < 2e-16 ***
freqnum      -2.475e-04  3.805e-05  -6.505 3.61e-10 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.0007255 on 277 degrees of freedom
  (70 observations deleted due to missingness)
Multiple R-squared: 0.5749,     Adjusted R-squared: 0.5687
F-statistic: 93.64 on 4 and 277 DF,  p-value: < 2.2e-16
```

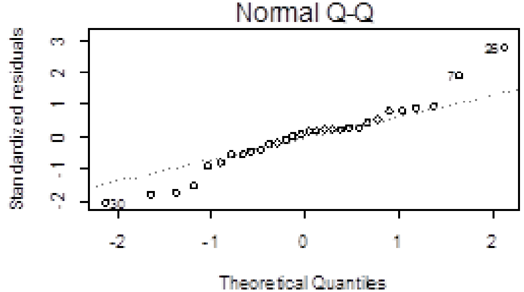


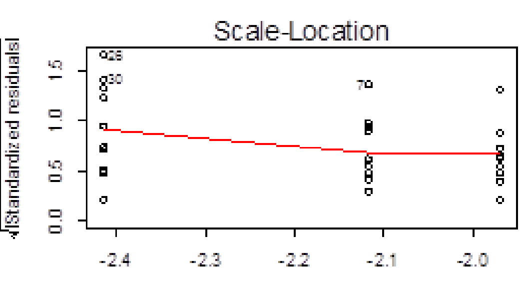
GLM 6: $\log_{10}$ (Variance in resistance to ceftriaxone) = Degree of heterogeneity in the antibiotic environment

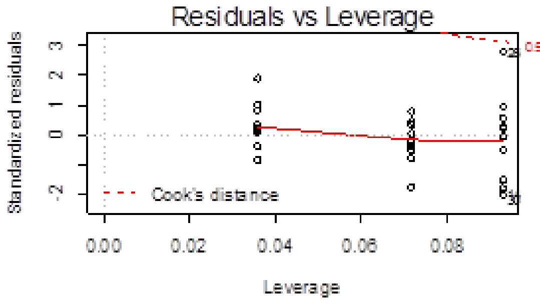
```
Analysis of Variance Table

Response: log10(cefx.variance)
                   Df Sum Sq Mean Sq F value    Pr(>F)
days.before.change  1 1.0394 1.03943  11.308 0.002249 **
Residuals          28 2.5739 0.09192
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
>
```





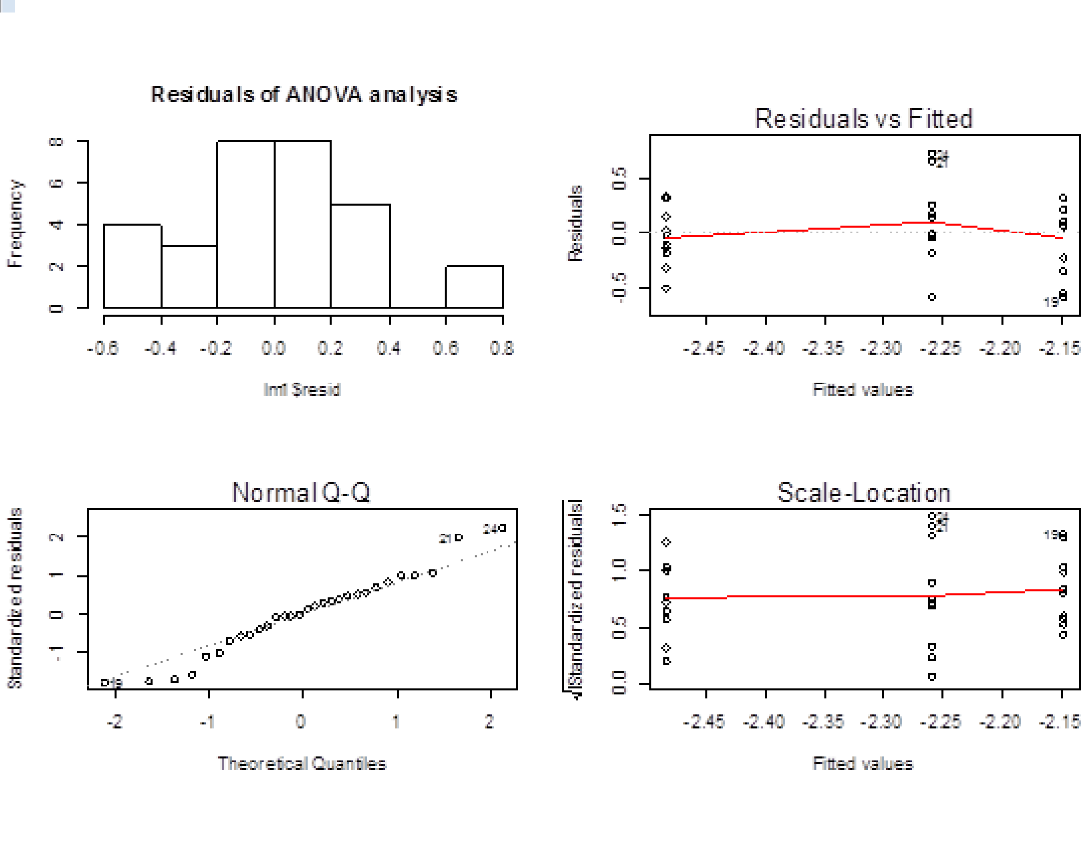


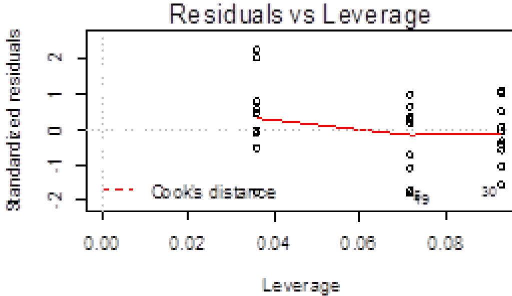




GLM 7: $\log_{10}$ (Variance in resistance to sulfamethoxazole) = Degree of heterogeneity in the antibiotic environment

```
Analysis of Variance Table

Response: log10(sulfa.variance)
                  Df Sum Sq Mean Sq F value Pr(>F)
days.before.change  1 0.5865 0.58650  5.4321 0.0272 *
Residuals         28 3.0231 0.10797
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
>
```





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