The evolution of antibiotic resistance in experimental populations of bacteria

by

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Abstract

Antibiotic resistance is a major threat to public health. Understanding how it evolves, and the genes that underlie resistance, is the main goal of my Ph.D. research. After a resistance mutation arises, it's fate within a pathogen population will be determined in part by its fitness: mutations that suffer little or no fitness cost are more likely to persist in the absence of antibiotic treatment. My research centers on understanding this process better by gaining knowledge about the spectrum of fitness effects associated with antibiotic resistance mutations.

Using a meta-analysis framework I find that, across a range of antibiotics and pathogens, on average single resistance mutations exhibit fitness costs in the absence of drug, however, there are instances of cost-free mutations. To evaluate the conditions leading to the persistence of resistance in the absence of antibiotic, I use experimental evolution of the opportunistic pathogen *Pseudomonas aeruginosa* and the antibiotic ciprofloxacin to investigate the phenotypic and genetic differences associated with constant and fluctuating drug treatment. I find that fluctuating drug treatment leads to the evolution of cost-free resistance. At the genetic level, cost-free resistance is the result of second-site mutations that compensate for the fitness cost associated with ciprofloxacin-resistance mutations. Further examination of the resistance mutations shows a lack of epistatic interactions between co-occurring mutations that confer resistance within a single isolate. To investigate the repeatability of the genetic causes of resistance, I execute a second evolution experiment using multiple clinical strains of *P. aeruginosa* adapting to a constant ciprofloxacin selective

pressure. I find a remarkable lack of parallel evolution at the genomic level both within and between different *P. aeruginosa* strains.

I have shown that antibiotic resistance is costly, and that these costs can be ameliorated by second-site mutations that readily arise over short time scales. Additionally, different strains of the same bacteria can gain resistance through a diverse set of genetic mutations. On an applied level these results are not positive; combating resistance evolution will be difficult because pathogens can easily compensate fitness costs of resistance, and resistance itself can be gained via a large number of genetic targets.

Résumé

La résistance aux antibiotiques est une menace sérieuse à la santé publique. Mieux comprendre comment cette résistance est évolue , et les gènes liées, est le bût de mon doctorat. Après qu'une mutation liées à la résistances est produits, sa destinée dans une population pathogène sera déterminée en partie par sa valeur adaptative darwinienne: les mutations qui exiges peut ou aucun coût adaptative sont plus probables à persister hors la traitements des antibiotiques. Ma recherche se concentre a mieux comprendre cette processus par améliorer nos connaissances de la gamme des effets adaptative associées avec les mutations liées à la résistance aux antibiotiques.

Utilisant une structure de meta-analyse je trouve que, à travers un assortiment d'antibiotiques et pathogènes, en moyenne une seule mutation démontre un coût adaptative hors la traitements des antibiotiques, tandis, il existe certaine cas de mutation sans coût. Pour évaluer les circonstances qui mènent à la persistance de la résistance aux antibiotiques hors traitements, j'utilise l'évolution expérimentale de la pathogène opportuniste *Pseudomonas aeruginosa* et l'antibiotique ciprofloxacin pour étudier les différences phénotypiques et génétiques associées à la traitement d'antibiotique constant et fluctuant. Je trouve que la traitement fluctuant mène a l'évolution des mutations de résistance sans coût adaptative. Au niveau génétique, la résistance sans coût adaptative est le résultat de mutations à des sites secondaires qui récompense pour le coût adaptative des mutations directement associées à la résistance de ciprofloxacin. L'examination supplémentaire des mutations associées à la résistance démontre aucune épistasie entre les mutations de résistances qui s'évolue dans la même génotype. Pour mieux comprendre la répétabilité des facteurs génétiques liées à la

résistance, j'ai faites une deuxième expérience d'évolution expérimentale avec plusieurs souches de *P. aeruginosa* sous traitement constant de ciprofloxacin. J'était étonnée par la manque d'évolution parallel au niveau génétique entre les copies exactes et entre souche différents de *P. aeruginiosa*.

J'ai pu démontrer que la résistance antibiotique est couteuse, et que ces coûts peuvent être améliorer par des mutations de site secondaire qui se produisent rapidement. En plus, des souches différents de la même bactérie peuvent accueillir la résistance par une gamme diverse de mutations. Au niveau d'application, ces résultats évoque de la concerne; combattre l'évolution de la résistance aux antibiotiques sera difficile parce-que les pathogens peuvent facilement compenser pour la coût de la résistance, et la résistance elle même peuvent être évolue par nombreuse cible génétiques.

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"Success consists of going from failure to failure without loss of enthusiasm."

- Winston Churchill

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DEDICATION

I would like to dedicate this thesis to my late grandmother. Though I lost my grandmother during the course of my Ph.D., the life she lived serves as inspiration for me to live to my fullest potential each day.

Finally, this is dedicated to my husband, Chris, my calm in the storm. Your patience, sacrifice and love allowed me to complete this degree. Thanks for being my biggest cheerleader and having more confidence in my abilities than I do myself.

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Chapter 1

An introduction to the evolution of antibiotic resistance

The introduction of antibiotics is likely the most important public health development of the twentieth century. However, the exhaustive use of antibiotics in healthcare and agriculture has greatly increased the frequency of resistance among human pathogen populations (Palumbi 2001; Perron et al. 2006). The World Health Organization released a report in 2012 (World Health Organization 2012) stating there is an imminent threat of a post-antibiotic era. Therefore understanding resistance acquisition and persistence is a pressing health concern.

One strategy for reducing the spread of resistance is to halt the use of antibiotics, eliminating the adaptive advantage of resistance. The implicit assumption underlying this strategy is that resistance is costly, such that resistant bacteria have reduced growth in the absence of antibiotic (Levin et al. 1997; Andersson and Levin 1999). However, resistance persists in the absence of antibiotic selection pressure (Enne et al. 2001; Sundqvist et al. 2010) and has been increasing worldwide (World Health Organization 2012). A critical parameter influencing the persistence of resistance is the fitness cost of resistance mutations. Fitness costs have been shown to play an important role in determining patterns of antibiotic resistance emergence and spread, as the most commonly occurring resistance mutations in clinical isolates are often those that incur the lowest fitness cost in the laboratory (Andersson 2006; Gagneux et al. 2006). However, we lack knowledge about the spectrum of fitness effects associated with antibiotic resistance mutations.

The general aim of my thesis is to examine how antibiotic resistance arises in pathogen populations, whether it is associated with a cost, and the underlying genomics of resistance. Answering these questions involves some of the most long-standing themes in evolutionary biology: understanding the rate and extent of adaptation, the variety of genetic targets underlying adaptation and the specificity of adaptation. These evolutionary processes can be observed directly using experimental evolution, the method of experimentally evolving organisms with short generation times in controlled environments. Experimental evolution has previously been used to provide answers to fundamental questions about the dynamics of adaptation and genome evolution (Barrick et al 2009), diversification (Rainey and Travisano, 1998) and speciation (Dettman et al. 2007). It has also been established as a methodology that can be used to investigate evolutionary trajectories of antibiotic resistance pathogen populations (Gifford et al. 2015; Kim et al. 2014; Vogwill et al. 2014; Gifford and Maclean 2013; Toprack et al. 2012; Wong et al. 2012; Lee et al. 2010). By pairing experimental evolution with next-generation whole genome sequencing and molecular techniques such as allelic replacements, I directly identify individual mutations responsible for resistance and measure their adaptive effects. In my thesis I use experimental evolution of the opportunistic pathogen *Pseudomonas aeruginosa*, adapting to the fluoroquinolone antibiotic ciprofloxacin. *P. aeruginosa* is the pathogen most commonly associated with mortality in cystic fibrosis (CF) patients (Corey & Farewell 1996; Rosenfeld et al. 1997; Canadian Cystic Fibrosis Foundation, 2011). Infections in CF patients are often treated with ciprofloxacin, and treatment often leads to high levels of resistance (Wu et al. 1999). Thus my experimental conclusions have direct clinical applications.

I begin this thesis with the question “Is antibiotic resistance costly?”. To answer this question I used a meta-analysis and measured the fitness costs of single mutations that confer

resistance (chapter 2), across a range of antibiotics and bacterial species. I found that resistance is generally costly, although there was a large amount of variation and there are occurrences of cost-free resistance mutations. These instances of cost-free resistance are especially important for the management of resistance. Therefore I designed an experiment to test the selection conditions that generate cost-free resistance. Chapter three examines the effect of different temporal drug regimes on resistance acquisition, examining adaptation at both phenotypic and genetic levels. Chapter four examines the specific mutations that conferred ciprofloxacin-resistance in chapter three, their individual and combined effects on fitness and resistance, and whether epistatic interactions occur between them. In chapter five I performed a second selection experiment to examine the repeatability of resistance evolution between different clinical strains of *P. aeruginosa*. I finish with a concluding chapter (chapter six), briefly summarizing the main findings of this thesis.

Much of the work described in this thesis was a collaborative effort, however I took a lead role on each of the projects. The details of my collaborator's contributions are described at the beginning of each chapter, where applicable.

Chapter 2

The fitness costs of antibiotic resistance mutations

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Collaborator contributions:

A. Wong performed and analyzed the competitive fitness simulations (Box 2.2).

Abstract

Antibiotic resistance is increasing in pathogenic microbial populations, and is thus a major threat to public health. The fate of a resistance mutation in pathogen populations is determined in part by its fitness: mutations that suffer little or no fitness cost are more likely to persist in the absence of antibiotic treatment. In this review we performed a meta-analysis to investigate the fitness costs associated with single mutational events that confer resistance. Generally, these mutations were costly, although several drug classes and species of bacteria on average did not show a cost. Further investigations into the rate and fitness values of compensatory mutations that alleviate the costs of resistance will help us to better understand both the emergence and management of antibiotic resistance in clinical settings.

2.1 Introduction

The initial optimism accompanying the introduction of antibiotics to control infection over 60 years ago has been steadily worn down by continuing reports of antimicrobial resistance (AMR) among nearly all human-associated pathogens (Palumbi 2001; Perron et al. 2006). AMR already represents a major burden on health care systems around the world: estimates of the economic burden of AMR are estimated to be at least 1.5 billion euros annually in Europe (World Health Organization 2012) and on the order of $200 million annually in Canada alone (Conly 2002), and these costs are expected to get worse with time.

Widespread therapeutic and prophylactic use of antibiotics in healthcare and agriculture constitutes a strong and persistent selective pressure favouring the evolution of antibiotic-resistant strains, a phenomenon characterized by Hall as 'use it and lose it' (Hall 2004). For this reason research has been increasingly focused on eliminating, or at least controlling, AMR once it has evolved. The most common strategy is to stop using antibiotics, the assumption being that mutations conferring resistance impose a large fitness cost in the absence of the drug. Note that fitness is taken here to be the rate of replication under prevailing environmental conditions, and can be measured through competitive fitness trials or as the growth rate of the strain or population being considered. Sensitive genotypes that do not pay a cost of resistance should therefore replace resistant strains at a rate proportional to the magnitude of the cost imposed by resistance (Levin et al. 1997; Johnsen et al. 2009).

Resistance mutations may be expected to impart a fitness cost because they target important biological functions in the cell (Table 2.1). For example, resistance to

fluoroquinonlones in Pseudomonads can cause impaired motility (Stickland et al. 2010), and resistance to aminoglycosides can alter the structure of the ribosome (Springer et al. 2001; Holberger and Hayes 2009**)** and so interfere with basic cellular functions.

Table 2.1. Antibiotics included in this meta-analysis.

Antibiotic class	Examples of antibiotics (used in this study)	Mode of action (target)	Mechanisms of resistance	Known genes involved in mutations conferring resistance
Alpha-pyrone	Myxopyronin	RNA transcription: inhibits bacterial RNA polymerase (RNAP)	Altered target	*rpoB, rpoC*
Aminoglycoside	Amikacin, streptomycin, spectinomycin	Protein synthesis: binds to 30S subunit bacterial ribosome inhibiting translation	Drug efflux, altered target, enzymatic inhibition of drug	*rpsL, rrs, rrl, rpsE*
Coumarin	Coumermycin, novobiocin	DNA replication: inhibits DNA gyrase and topoisomerase IV enzyme	Drug efflux, altered target	*gyrB*
Dihydrofolate reductase inhibitor	Trimethoprim	DNA replication: blocks the folate coenzyme biosynthetic pathway, essential for providing monomers for DNA synthesis	Decrease thymidine requirement, altered target	*dfrA*
Fusidane	Fusidic acid	Protein synthesis: prevents the turnover of elongation factor G from the ribosome	Drug efflux, mutations in elongation factor G	*fusA*
Macrolide	Clarithromycin, erythromycin, tylosin	Protein synthesis: binds to the 50S subunit bacterial ribosome inhibiting translation	Drug efflux, altered drug target, inactivation of drug, mutations in 23S rRNA	23S rRNA genes
Quinolone	Ciprofloxacin,	DNA replication:	Drug efflux,	*gyrA, gyrB,*

	nalidixic acid, norfloxacin	inhibits bacterial DNA gyrase and topoisomerase IV enzyme	altered target	*parC, parE, grlA*
Rifamycin	Rifampicin	RNA replication: binds to RNA polymerase	Altered target	*rpoB*

Bryskier (2005); Andersson and Hughes (2010); Davies and Davies (2010); Walsh (2013).

Clinical and epidemiological evidence on the effectiveness of stopping antibiotic treatment as a strategy for reducing resistance is both limited and mixed. Clinical studies have shown that in some cases resistant bacteria remained abundant in the population (Sundqvist et al. 2010) or even increased in frequency (Enne et al. 2001; Arason et al. 2002) despite the absence of drug, while in others the proportion of resistant bacteria within the population declined (Seppala et al. 1997; Austin et al. 1999; Bergman et al. 2004; Gottesman et al. 2009), as expected. In epidemiological studies, reducing the use of antibiotics often leads to a reduction in the frequency of resistant strains, but it rarely succeeds in eliminating them altogether (Salyers and Amabile-Cuevas 1997; Andersson 2003; Enne 2010; Johnsen et al. 2011).

Evidently, the strategy of stopping the use of a drug once resistance has evolved is not always effective at eliminating resistance. The question is, why? Three hypotheses could account for the persistence of AMR strains in the absence of antibiotic (Andersson 2003; Andersson and Hughes 2010). First, genetic linkage between the resistance gene(s) of interest and selected loci may lead to genetic co-selection (Borrell et al. 2013) and prevent the elimination of resistance. Many instances of persistence in multi-drug resistant strains or plasmid-mediated resistance are likely due to this mechanism. Second, the fitness costs incurred by resistance mutations may be compensated by second-site mutations that increase

fitness without compromising resistance. Such compensatory evolution has been observed in both *in vitro* (Levin et al. 2000), *in vivo* (Bjorkman et al. 2000) and in clinical studies (Nagaev et al. 2001; Bjorkholm et al. 2001; Gagneux et al. 2006; Comas et al. 2011). Third, the pleiotropic costs of resistance among mutations may be so highly variable as to sometimes include 'no-cost' mutations **(**Sander et al. 2002; Ramadhan & Hegedus 2005**)**, those that have fitness indistinguishable from (or even greater than) their antibiotic-sensitive ancestor in the absence of antibiotic. This last hypothesis has proven challenging to evaluate because we know very little about variation in costs of resistance among different genetic targets. Previous work has shown that costs of resistance among single-step, chromosomal mutations can be highly variable (Kassen and Bataillon 2006) and the literature contains a number of reports of putatively cost-free mutations, including streptomycin resistance in the *rpsL* locus of *Mycobacterium smegmatis* (Sander et al. 2002), isoniazid resistance in *katG* of *Mycobacterium tuberculosis* using a mouse model (Pym et al. 2002) and quinolone resistance in *gyrA* and *parC* of *Streptococcus pneumoniae* (Gillespie et al. 2002).

Box 2.1. Mechanisms of gaining and maintaining antibiotic resistance.

Prokaryotic microbes can gain resistance de novo by adaptive evolution, or via horizontal gene transfer of resistance cassettes between microbes. Resistance can be maintained, in the absence of antibiotic selection, in three ways. Resistance mutations may incur no fitness costs, and thus remain in the population in the absence of antibiotic selection pressure. Alternately, costs of resistance can be compensated via second-site mutations that restore organismal fitness in the absence of antibiotic selection. Finally, genetic co-selection can occur whereby there is a genetic linkage between a resistance-conferring gene and either

other selected genetic markers or other selected resistance mutations to different antibiotics, thereby enabling non-selected resistance to remain within the population.

To explore the nature of the variation in fitness costs among resistance mutations in more detail we collate data from the literature on the fitness effects of single chromosomal mutational events that confer antibiotic resistance from a wide range of pathogenic bacterial species. Our objective is to examine the prevalence of so-called 'no-cost' resistance mutations with the aim of evaluating whether these could make a substantial contribution to the persistence of AMR. We focus on studies that measure fitness directly through competitive assays between a strain with a resistance mutation and the isogenic strain lacking that mutation. This method is preferred over alternatives such as the measurement of population growth rates in pure culture because it is an integrated measure involving all phases of the growth cycle and can capture aspects of competition such as toxin production that may not be reflected in pure culture assays.

Table 2.2 Glossary.

Compensatory mutation: A second-site mutation that occurs after a mutation that confers resistance, which lessens or alleviates the fitness costs associated with resistance.
Cross-resistance: The propensity of a genetic change that confers resistance to one drug also to affect resistance to a different drug (by either increasing or decreasing resistance)
Epistasis: When the fitness effect of a mutation is modulated by its interactions with other

genes or mutations in the genome

Genetic co-selection: The occurrence of genetic linkage between the resistance-conferring gene and other selected genetic markers. Thus, even though a non-selected resistance gene might confer a cost it could remain in the population because of its genetic linkage to a second marker.

Genetic plasticity: The alterable nature of prokaryotic genomes that enables the fluid exchange of DNA from one microorganism to another.

Horizontal gene transfer: The acquisition of a gene by a means other than direct inheritance from a parent cell (vertical transfer). Common in many bacteria and archaea, mechanisms of horizontal gene transfer include transformation, conjugation and transduction.

Minimum inhibitory concentration: The lowest concentration of an antibiotic that will inhibit the visible growth of a microorganism after overnight incubation.

Relative fitness: The capability of a genotype or individual to survive and reproduce in comparison with a second genotype or individual

Previous work has highlighted the potential importance of no-cost resistance mutations, and the variation in costs of resistance more generally, in pathogenic bacteria (Andersson 2003; Andersson 2006; Andersson and Hughes 2010). To our knowledge no formal meta-analysis

on the relative costs of antibiotic resistance mutations has been performed. In this article we analyze 179 mutations (121 unique), comprising 8 bacterial species and 16 antibiotics, and address the following questions: Are certain antibiotics or species more likely to be associated with no-cost resistance? If so, why? Is there a correlation between the magnitude of the fitness cost and the level of resistance conferred by a given mutation? If higher levels of resistance require a cell to devote more resources to detoxifying or eliminating a drug, or involve mutations of greater phenotypic effect, we might expect a negative relationship between MIC and fitness. However, this hypothesis has rarely been tested directly. Answering these questions provides a glimpse into some of the most basic patterns associated with resistance mutations and their effects on fitness, a subject that has received surprisingly little direct attention in the literature.

2.2 Materials and Methods

We identified suitable studies to include in our dataset by searching the online database Web of Science with the keywords "antibiotic resistance" + "fitness cost" published as of November 2013. Additional studies were found by searching the reference sections of these articles. Many studies could not be included because fitness was measured as the growth rate of each strain rather than via competitive fitness assays.

The principle behind a competitive fitness assay is that a fitter type will outcompete a less fit type when co-cultured in the same set of growth conditions. The rate at which one type excludes the other is a measure of its fitness. Estimating competitive fitness requires monitoring the change in relative frequencies of otherwise isogenic sensitive and resistant

strains over time. Different research groups use slightly different methods to calculate fitness, so here we have recalculated all fitness estimates in terms of the Malthusian growth parameter to facilitate direct comparisons (see Box 2.2).

To be included in our database, an article had to satisfy strict selection criteria: (1) data needed to include an estimate of both mean and variance of competitive fitness; (2) competitive fitness had to be measured via *in vitro* assays; (3) resistance had to be conferred by a single mutational event; and (4) the study needed to be performed in bacteria. We thus rejected many studies that did not include the relevant measures. Altogether, 24 studies were included in the analysis comprising 16 antibiotics (Table A.1) from 8 antibiotic classes. These studies further included a total of 8 bacterial species (Table A.2**)** including *Escherichia coli*, a ubiquitous Gram-negative bacterium, and *S. pneumoniae*, an important Gram-positive opportunistic pathogen. Four papers were excluded because replicate measures of fitness were not provided, making it impossible to estimate the variance in fitness (Billington et al. 1999; Binet and Maurelli 2005; Enne et al. 2005; Nessar et al. 2011). Four further papers were excluded because competitive fitness was calculated *in vivo*, which is not a comparable metric to relative fitness calculated *in vitro* because it lacks a measure of generation time (Nagaev et al. 2001; Gustafsson et al. 2003; Luo et al. 2005; Luangtongkum et al. 2012). Two papers were excluded because relative fitness measures were illustrated graphically, without providing the numerical measures necessary for the meta-analysis (Bjorkholm et al. 2001; Huitric et al. 2010).

Box 2.2 Estimation of competitive fitness

Competitive assays provide the "gold standard" for measuring fitness. In its simplest form, a

competition experiment allows for the estimation of fitness for a focal strain (either a single genotype or a population) relative to a defined, typically "wild-type" competitor, in a given laboratory environment. The focal strain and the wild-type competitor need to be readily distinguishable, for example via a phenotypic marker (lacZ+ vs lacZ-, or alternative fluorescent markers) or by genotyping. In order to estimate the costs of antibiotic resistance, the competition environment should be antibiotic-free, and is typically a standard laboratory medium such as LB (Lysogeny Broth). The focal strain and the wild-type are competed together for a fixed period of time, often 24 hours, and samples taken at the beginning and end of the competition allow the researcher to determine the number of focal and wild-type cells in the population. Fitness of the focal strain can then be inferred from the change in its relative abundance: If the focal strain suffers a fitness cost, then its frequency will decrease. Several formulae have been proposed for estimating the selection coefficient on a genotype, s, from competition data (where relative fitness is given by $1+s$). Lenski and colleagues (1991) consider fitness in terms of a Malthusian growth model, where the growth parameter for a strain is the number of doublings that it experiences over a given period of time. As such, the selection coefficient on the focal strain is defined as

$$s_l = \frac{\#\text{ of doublings of focal strain}}{\#\text{ of doublings of wild-type}} - 1 \tag{2.1}$$

Note that s_l is a unit-less parameter.

Alternatively, Dykhuisen and Hartl (1983) estimate the selection coefficient as

$$s_d = \left(\ln\left(\frac{n1f}{n1i}\right) - \ln\left(\frac{n2f}{n2i}\right)\right) / (\#\text{ of generations}) \tag{2.2}$$

where $n1_f$ and $n1_i$ are the numbers of cells of the focal strain at the end and the beginning of the assay, and $n2_f$ and $n2_i$ are the numbers of cells of the wild-type strain at the end and the beginning of the assay. Note that s_d has units generations^{-1}.

Both estimates of fitness are widespread in the literature, and we see no principled reason to prefer one to the other. In the context of the current meta-analysis, and more broadly, it is important to know the relationship between these two fitness estimators: to what extent do they agree in terms of the magnitudes of s? As such, we simulated pair-wise competitions using a simple growth model, in which each genotype grows according to a Poisson process. Samples were drawn from the simulated competition experiments, and s_l and s_d were estimated from the same data.

Notably, the magnitude of s_l is systematically larger than the magnitude of s_d. Figure 2.1 shows the relationship between s_l and s_d for a set of simulations where growth rates of the competing strains varied from 0 to 0.25, with the initial frequency of the focal strain set to 0.5, and competition carried out over 6 generations. Note that there is a tight linear relationship between s_l and s_d, with s_l exceeding s_d by a factor of about 1.7. The slope of the regression line appears to be insensitive to starting frequency, and is weakly affected by the number of generations of competition: So long as the competition experiment proceeds for 4 or more generations, s_l exceeds s_d by a factor of 1.7.

Given that competitive fitness is quantified fairly easily in bacteria, and is such an inclusive fitness measure, it was surprising to us that more studies have not employed this method. Many other studies investigating costs of resistance used growth rate as a proxy for fitness, which, as outlined above, incorporates only a single component of bacterial fitness. Including growth rate studies would have increased the sample size of our meta-analysis,

however, the inclusion of such data would reduce the clarity of the analysis because measures of growth rate and competitive fitness are poorly correlated. To examine this relationship in more detail for our dataset, we examined 35 mutations that supplied estimates of competitive fitness and for which we could compute a relative doubling time (Gillespie et al. 2002; Hurdle et al. 2004; Mariam et al. 2004). The data included 35 different antibiotic resistance mutations from three different bacterial species and four different antibiotics. In these studies competitive fitness and relative doubling time were not correlated (r^2=0.00734, p=0.619). We encourage future studies investigating costs of resistance mutations to use competitive assays to measure the relative fitness of resistant and sensitive strains.

For the purposes of this study we focused on resistance caused by single mutational events. The rationale behind this is simple: we need to be confident that resistance, and any associated fitness cost, is due to that mutation only and not other, co-occurring mutations. In our study, the vast majority of these mutations are single nucleotide polymorphisms (SNPs) although three small (3, 9 or 13 nucleotides, respectively) deletions were also included. All are chromosomal mutations because our interest is in the effects of these mutations on the fitness of the bacterial genotype itself, not the fitness of a plasmid on which the mutation arises. Thus we excluded studies that examined resistance gained by plasmids, horizontal gene transfer, those examining multidrug resistance, and studies which examined the fitness costs of multiple resistance mutations (e.g. Hurdle et al. 2004) within the same genetic background.

We report measures of drug resistance as the fold-increase in minimum inhibitory concentration (MIC) relative to the drug-sensitive ancestor. If absolute drug concentration was reported then these data were converted to fold-increase in MIC by dividing the concentration of drug required to inhibit growth in the resistant strain by the concentration of

drug required to inhibit growth in the sensitive ancestor. If MICs were reported as a greater-than value, the numerical value itself was recorded.

We analyzed the dataset using a random effects meta-analysis, using the *metagen* function within the meta package of R (Schwarzer 2013; R Development Core Team 2013). A random effects model is more appropriate than a fixed effects model as we cannot be certain that all studies included in our meta-analysis have equal variances. A relative fitness value greater than one indicates the mutation is both resistant and beneficial relative to the isogenic strain lacking the mutation; values less than one indicate the resistant mutation is costly. We evaluated the statistical significance of our fitness estimates by examining the 95% confidence intervals of the mean relative fitness: if these did not overlap with one, the fitness estimate was considered significant. Q-statistics were used to examine the heterogeneity of relative fitness values among groups. Each mutational event conferring resistance is taken to be a single unit of analysis, the rationale being that single mutational events occur independently of each other and often in different genetic backgrounds.

2.3 Results

2.3.1. Are there costs of resistance?

We found a significant fitness cost of resistance mutations (mean fitness=0.880, $z=129.3$, $p<0.0001$). The data exhibited significant total heterogeneity in their response ($Q_{total}=22966$, $p<0.0001$), indicating the presence of further explanatory variables within the dataset.

To describe the data in more detail, we plotted the standard error of mean fitness against mean fitness for all mutations in our collection (Figure A.1). A linear regression testing for funnel plot asymmetry is significant (t_{177}=-4.48, p<0.001), indicating bias in the data towards, in this case, costly mutations. The most parsimonious interpretation is that resistance mutations are often genuinely costly. It seems unlikely that this result represents a publication bias because, if anything, the observation of no-cost mutations is the more novel result. Notably, there is also substantial variation in costs of resistance with at least some mutations exhibiting little or no cost.

2.3.2. Variation in costs of resistance

Variation in costs of resistance can arise either because some mutations are costly and others are not, irrespective of the genetic background in which they occur, or because a given mutation is not costly in some genetic backgrounds but is costly in others. We investigated these alternatives by repeating our analysis with drug class, drug, or species as explanatory factors. A main effect of species indicates that the fitness effect of a given mutation depends on the genetic background in which it occurs while main effects of either drug or drug class indicate that the mutations themselves differ in their costs, independent of genetic background. We found evidence to support both explanations. There was a significant difference in the fitness costs of resistance mutations between drug classes (Figure A.2, $Q_{between}$=144,p<0.0001), between different drugs (Figure 2.2, $Q_{between}$ =282, p<0.0001), and between different bacterial species (Figure 2.3, $Q_{between}$ =75.5, p<0.0001). The mean relative fitness of resistance mutations associated with each of these different subgroups can be seen in Figures 2.2, 2.3 and Figure A.2 respectively. This result must be interpreted with caution,

however, because the data set is severely unbalanced: most resistance mutations are unique to a particular species and drug. Some of the variation is likely attributable to a lack of data, for example there are only four resistance mutations associated with fusidic acid (Table A.1), while other sources of variation might be because there is only a single drug-bacterium comparison for a given species, for example all eleven mutations measured for *M. tuberculosis* confer resistance to rifampicin (Table A.2). Nevertheless, our data suggest that costs of resistance can be highly variable and can depend on the class of drug used, the mutation itself, and the species within which that resistance mutation occurs.

Notably, some mutations appear to be either genuinely cost-free or the costs are so small they cannot be detected in these assays. In our data set, these putatively 'no-cost' mutations are associated with resistance to fusidanes and dihydrofolate reductase inhibitors (Figure 2.2, Figure A.2). Of note, the mean fitness of mutations conferring resistance to the dihydrofolate reductase inhibitor trimethoprim was greater than 1 (Figure 2.2), indicating that these resistance mutations are beneficial in the absence of drug. Additionally, resistance mutations in two species, *Enterococcus faecium* and *Borrelia burgorferi*, showed no evidence for a cost of resistance on average while those recovered from all other species were on average costly (Figure 2.3). The average fitness of each species and antibiotic comparison yielded no clear patterns in costs of resistance (Figure 2.4).

The sparseness of our data set precludes us from doing a fully factorial analysis of mutations, drugs, and species. However we can perform such an analysis for a subset of our data, namely, the resistance mutations associated with quinolone and rifamycin drug classes for both *Staphylococcus aureus* and *E. coli* (Table A.3). The simplest meta-analysis shows a significant fitness cost of resistance mutations (mean fitness=0.874, $z=64.4$, $p<0.0001$), with significant heterogeneity ($Q_{total}=11592$, $p<0.0001$). The fully factorial linear model that

treats species and drug class as fixed effects revealed a significant interaction between drug class and species ($F_{3,80}$=14.5, p<0.0001) but no significant differences associated with the main effect of species or drug class. Whilst this result is based on a limited data set, it does lend support to the idea that the fitness effect of resistance mutations in the absence of drugs depends on both the drug class and the genetic background in which those mutations appear.

The cell wall is an important target of mutations that confer antibiotic resistance and thus intuitively it seems possible that there may be a difference in fitness costs associated with antibiotic resistance between Gram-positive, that has a much thicker layer of peptidoglycan in their cell wall, and Gram-negative bacteria, that has a much thinner cell wall. Gram-positive bacteria had a significantly greater fitness costs associated with resistance mutations (mean fitness=0.822) when compared with Gram-negative bacteria (mean fitness=0.973, t_{156}=-5.19, p<0.0001). Again, caution must be used in interpreting this result because there were a greater number of Gram-negative than Gram-positive bacteria in our dataset.

2.3.3. The relationship between MIC and cost of resistance

We regressed MIC against relative fitness to test the prediction that high levels of resistance impose greater fitness costs than lower levels of resistance. The relative fitness of a resistance mutation is negatively correlated with the fold-increase in MIC conferred by the mutation (Figure 2.5, t_{126}=-6.21, p<0.0001), with fold-increase in MIC accounting for 22.8% of the variation in fitness ($F_{1,126}$=38.6, p<0.0001). A subset of data was used for this analysis because 7 studies did not measure MIC (Schrag and Perott 1996; Criswell et al. 2006; Gagneux et al. 2006; Balsalobre and de la Campa 2008; Hao et al. 2009; Trinidade et al.

2009; Borrell et al. 2013). Although it would be of interest to perform separate regressions for each class of antibiotic to see if the correlation holds across different mechanisms of action, for many of the drug classes sample sizes are too small to permit reliable regression coefficients to be estimated.

2.4 Discussion

Using meta-analysis we have found that most resistance mutations in bacteria confer a fitness cost. This result is not surprising as many antibiotics target important cellular processes and resistance to them either disrupts those processes or imposes large energetic burdens that reduce competitive ability against sensitive strains. However, we have also found that there is substantial variation in fitness costs among species and drugs. This variation is large enough to include, occasionally, what might be classified as no-cost resistance mutations. This class of resistance mutation is not common, at least in our data set, and the actual cost associated with a particular mutation can depend on the genotype in which the mutation occurs. Costs will also likely depend on the environment in which the genotype is growing due to variation in resource identity and abundance, as well as the general level of stress imposed on the cell. We cannot examine this hypothesis in more detail, unfortunately, as the appropriate data is not available. Furthermore we know little about how well measures of costs *in vitro* correlate to those incurred *in vivo*. Nevertheless based on the available data we do have we conclude that no-cost resistance mutations are likely not major contributors to persistent drug resistance in the absence of antibiotic.

Can we move from a phenomenological description of the variation in costs to a more mechanistic interpretation? Why, for example, are resistance mutations costly in some situations and not in others? Clearly, epistasis can play a role. In an evolutionary context, epistasis refers to a situation in which the fitness effect of a mutation depends on its genetic background. For example, ciprofloxacin resistance in *Campylobacter jejuni* is often via mutations in *gyrA*, which encodes a DNA gyrase. In one case, a resistance mutation (C257T) that showed a fitness benefit in one strain of *C. jejuni* was costly in another strain (Luo et al. 2005). Environment can also be important. The fitness estimates we examined here are all obtained *in vitro* and may not always reflect key aspects of fitness *in vivo*. Indeed, at least two resistance mutations in *fusA* that each confer resistance to fusidic acid in *Salmonella typhimurium* (Macvanin et al. 2004; Macvanin et al. 2003), and 2 mutations in *rpoB* (S464P and S531L) conferring rifampicin resistance in *S. aureus* show evidence that fitness costs change depending on whether it is measured *in vitro* or *in vivo* (Yu et al. 2005; Gagneaux et al. 2006).

Further generalizations are difficult because our data set is quite sparse. Most of the fitness estimates for a given mutation are gathered in single environments, which makes generalization difficult. Moreover, the fitness cost of the same resistance mutation is rarely assayed in more than one strain, let alone more than one species. Thus our ability to draw strong inferences on the causes of such variation in costs of resistance remains limited.

Nevertheless, at least two results appear noteworthy and warrant further investigation. The first is that there is fairly good evidence that mutations that confer larger MICs are more costly. This result is in line with previous studies that found a similar relationship between MIC and growth rate (Ender et al. 2004; Hurdle et al. 2004). It has also been shown that the first mutations that arise and confer resistance to ciprofloxacin in *Pseudomonas aeruginosa*

are generally costly (Wong et al. 2012). This relationship can be understood very generally in terms of Fisher's geometric model of adaptation: mutations of large effect for one phenotype (MIC) have pleiotropic effects on other phenotypes, the result being that an individual is knocked off a local fitness peak. More mechanistically, the causes of pleiotropy due to resistance mutations stem either from the fact that dealing with high levels of toxin in the environment is an energetically costly process that takes resources away from other cellular functions, or because resistance is gained via mutations that alter or disrupt enzyme function and the production of essential proteins.

The second notable result is that the presence of a thicker cell wall, one of the defining features of Gram-positive bacteria, is associated with larger fitness costs. While this result must be interpreted with caution because it is based on few data points, it is interesting that it holds for many different kinds of resistance mutation, including those that confer resistance through both small molecule efflux and target binding. It thus appears to be a very general result although the biological reason for this remains unclear. One, rather simplistic, suggestion is that the presence of a cell wall imposes an additional energetic burden on toxin clearance that Gram-negative bacteria do not have to deal with.

The main clinical implication of this work is that no-cost mutations are probably not a common reason why antibiotic resistance persists in the absence of drug use. Rather, it seems much more likely that persistence is due either to co-selection of genetically linked mutations or because the fitness cost of resistance mutations are often compensated by mutations elsewhere in the genome. Indeed, previous work suggests that compensatory mutations can arise within a few generations following the emergence of resistance (Bjorkman et al. 2000; Maisnier-Patin et al. 2002; Kugelberg et al. 2005; Paulander et al. 2007; Bataillon et al. 2011; Sousa et al. 2012; Wong et al. 2012; de Vos et al 2013). Furthermore, the presence of

additional resistance mutations can compensate for the cost of an initial resistance mutation, a form of positive epistasis for fitness (Trinidade et al. 2009).

That said there is some evidence that resistance mutations with low or no fitness costs can be prevalent in clinical populations. For example the spectrum of mutations in *rpoB* that cause rifampicin resistance in clinical isolates of *M. tuberculosis* and *S. aureus* are biased in favour of low-cost mutations (O'Sullivan et al. 2005; O'Neill et al. 2006). The K424R substitution in the 30S ribosomal protein S12 also does not exhibit a fitness cost associated with resistance in both *S. typhimurium* and *M. smegmatis*, and this same mutation is also primarily responsible for resistance to streptomycin in clinical isolates of *M. tuberculosis* (Bottger et al. 1998; Sander et al. 2002). Other mutations in *rpoB* can be costly, as evidenced by the fact that putative compensatory mutations in *rpoA* and *rpoC* are routinely isolated alongside some resistance-causing *rpoB* mutations (Comas et al. 2011). Thus, while no-cost mutations may not be a general explanation for why antibiotic resistance persists in the absence of drug, it may be important in specific cases.

Taken together, these observations suggest that no-cost mutations cannot be automatically dismissed as an explanation for why antibiotic resistance persists in clinical settings even after the offending drug is removed from use. Whether costs of resistance are an effective guide to predicting the prevalence of resistance following reduced drug prescription remains an open question. Enne (2010) investigated this question directly and found mixed results, with reduced prescriptions leading to reduced prevalence in some cases but not others. Notably, in two cases where our results indicate a significant cost of resistance for a given bacterial species, Enne also found that prevalence was reduced following prescription reduction of penicillin for *S. pneumoniae* (Austin et al. 1999) and quinolones for *E. coli* (Gottesman et al. 2009), lending some support to the predictive ability

of costs of resistance from individual mutations. However other examples show contrasting results. For example, persistent quinolone resistant *E. coli* was found in a remote community where quinolones were not prescribed (Pallecchi et al. 2012), suggesting co-selection. It has been suggested that, in the case of quinolone resistance in this species, co-selection could occur because resistance can be plasmid-mediated (Wang et al. 2003).

The take home message here is that there may not be any simple connection between the cost of resistance for individual mutations and clinical prevalence of resistance. Given the variety of factors that can modulate costs – epistasis between the resistance mutation and genetic background or even other resistance mutations, the environment, the occurrence of compensatory mutations and genetic linkage between the resistance mutation and other mutations under selection – this should not be surprising. The evolution of costs of resistance and its connection to clinical treatment is a more complex issue than was initially thought. Future work should focus on disentangling the contributions of these various factors, in particular compensatory mutations that seem to evolve very quickly alongside costly resistance mutations, to the persistence of antibiotic resistant strains in clinical and environmental settings.

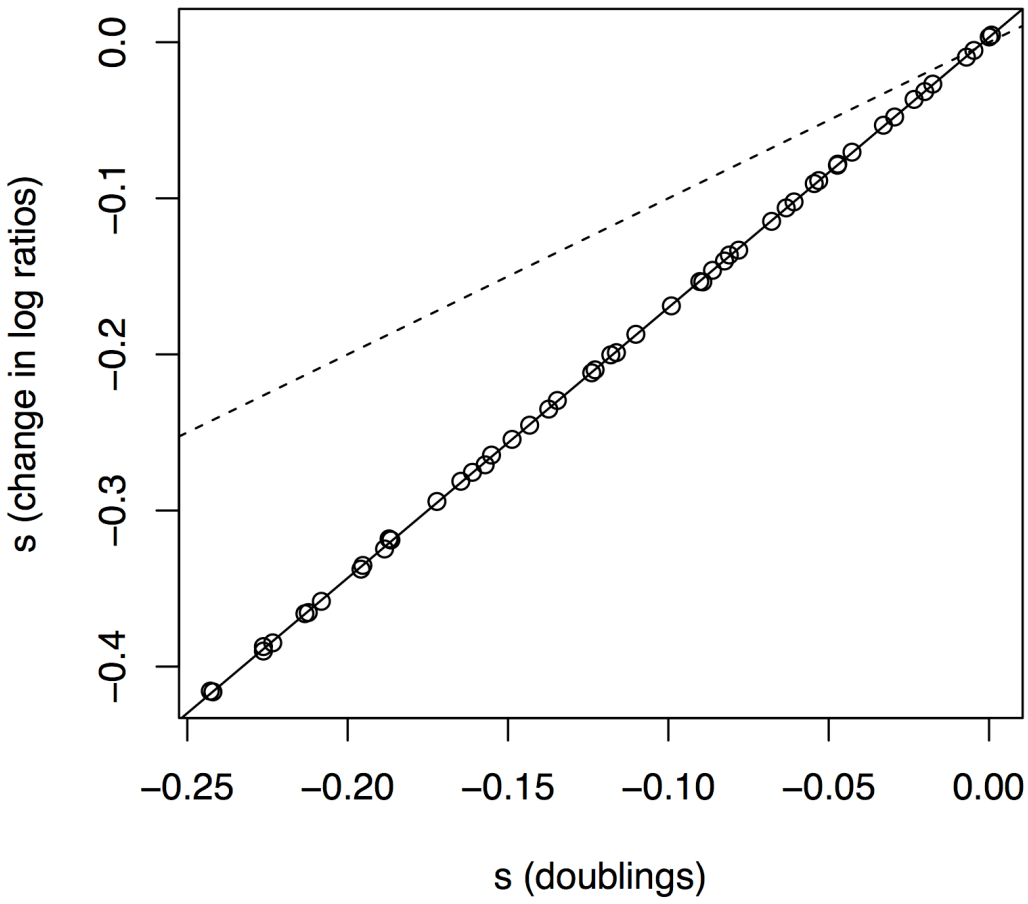

Figure 2.1. Estimation of selection coefficients using the Lenski and Dykhuisen estimators (Box 2.2). Competition experiments were simulated for two strains, with the wild-type strain doubling in each time unit, and the growth rate of the focal strain reduced compared to wild type by 0 to 0.25. Competition lasted six generations, starting with a 50:50 ratio of the two strains and an initial population size of 1 million. Each data point represents the mean values of s for 100 simulations. For each replicate, an average of 100 individuals were sampled and used to calculate s_l (y-axis) and s_d (x-axis). The dashed line represents a 1:1 relationship, and the solid line gives the linear regression of s_l on s_d .

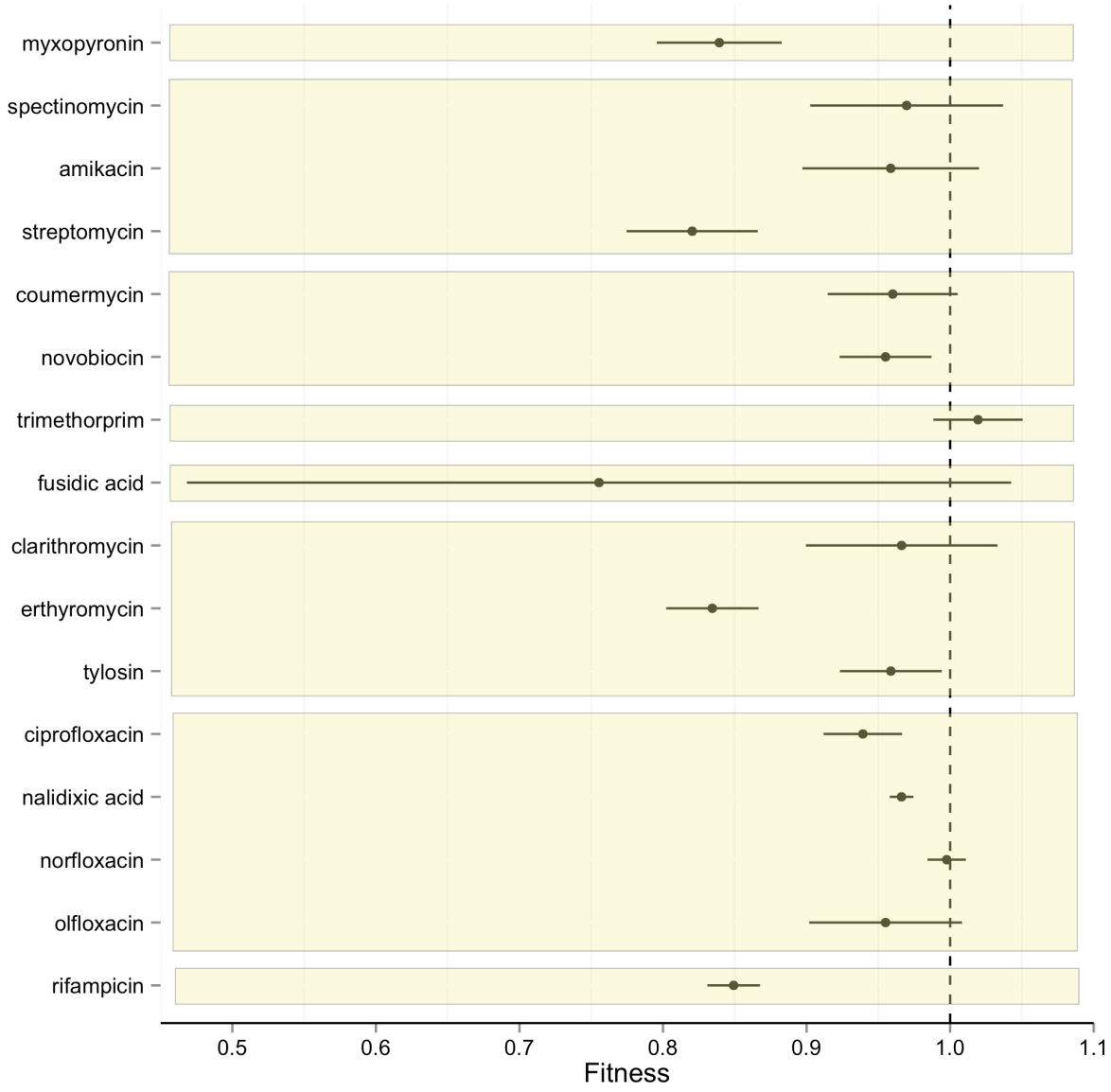

Figure 2.2. The mean relative fitness and 95% confidence intervals of antibiotic resistance mutations associated with a given antibiotic, grouped by class of antibiotic (from top to bottom alphay-pyrone, aminoglycoside, coumarin, dihydrofolate reductase inhibitor, fusidane, macrolide, quinolone and rifamycin). A fitness value of <1 indicates a fitness cost in the absence of antibiotic.

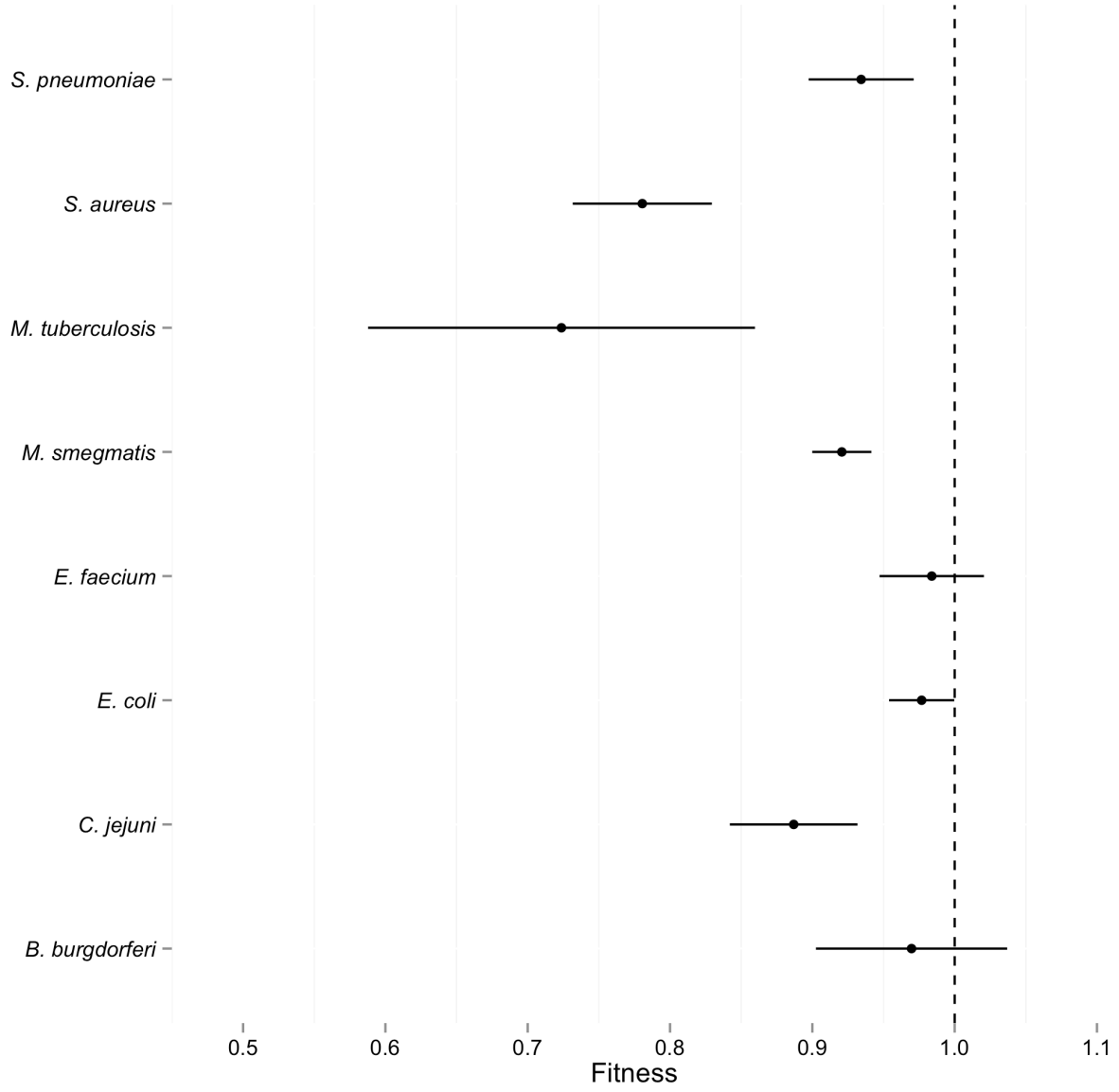

Figure 2.3. The mean relative fitness and 95% confidence intervals of antibiotic resistance mutations associated with a given species. A fitness value of <1 indicates a fitness cost in the absence of the antibiotic.

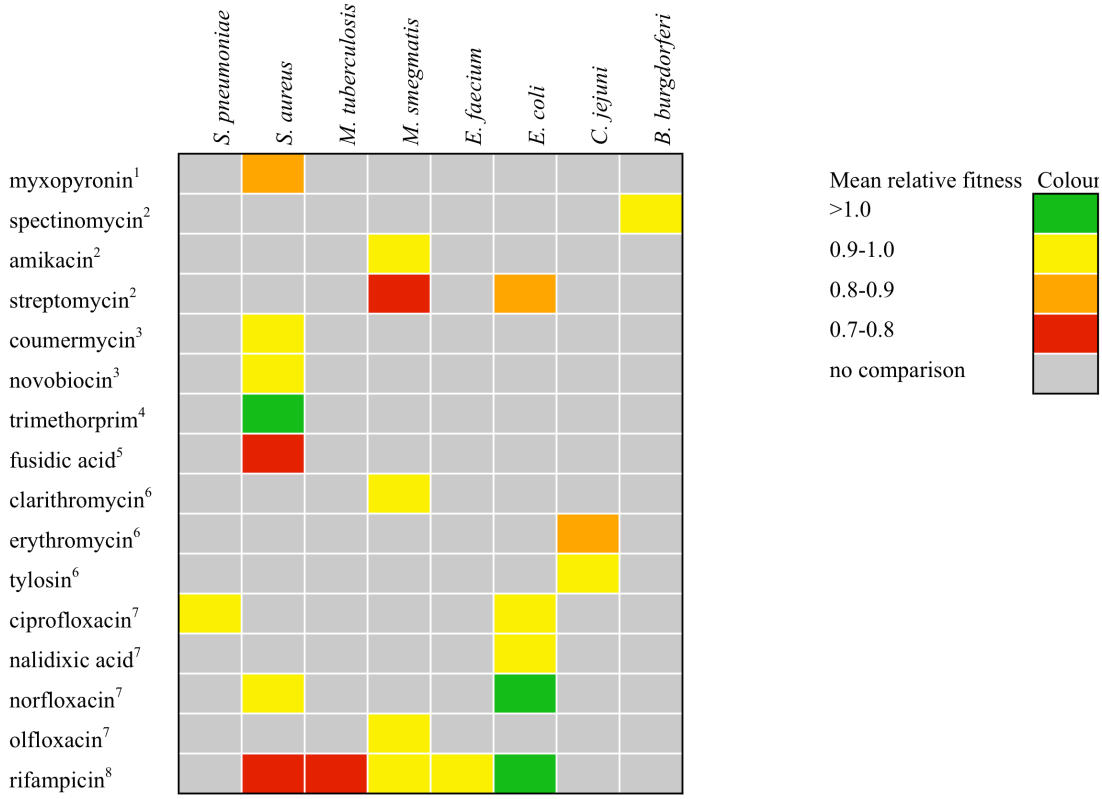

Figure 2.4. Species-antibiotic comparisons of the mean relative fitness of resistance mutations in the absence of antibiotic. Superscript numbers indicate antibiotic class: 1- alpha-pyrone, 2- aminoglycoside, 3- coumarin, 4- dihydrofolate reductase inhibitor (DHRI), 5- fusidane, 6- macrolide, 7- quinolone, and 8- rifamycin.

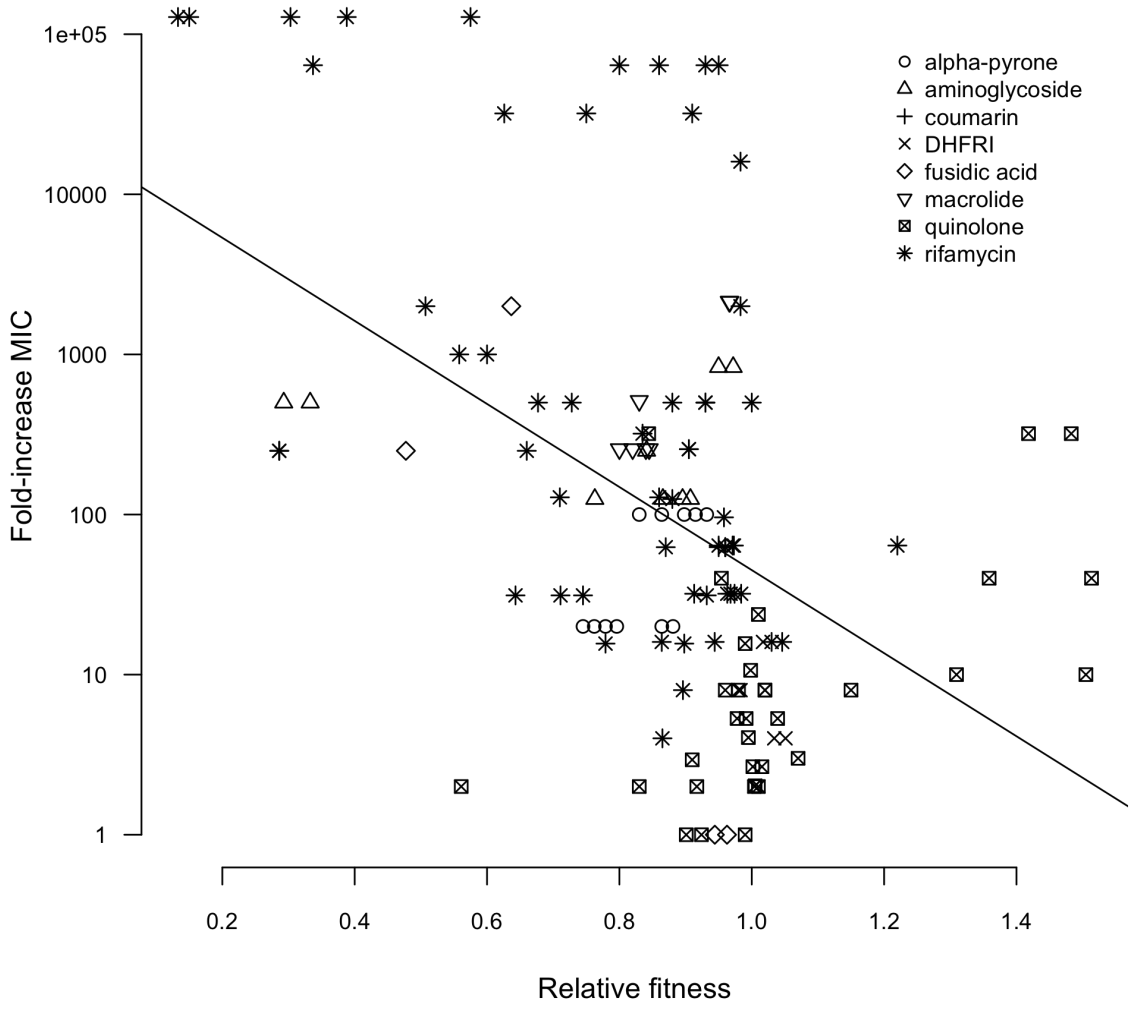

Figure 2.5. The level of resistance conferred by a mutation is negatively correlated with its fitness in the absence of antibiotic ($r^2 = 0.228$). Different symbols are associated with different classes of antibiotic.

Chapter 3

Evolution of cost-free resistance under fluctuating drug selection in *Pseudomonas aeruginosa*

This chapter is reproduced from:

Melnyk, A. M., and R. Kassen. (in prep. for submission to *PLoS Pathogens*) "Evolution of cost-free resistance under fluctuating drug selection in *Pseudomonas aeruginosa*."

Abstract

Antibiotic resistance evolves rapidly in response to drug selection but it can also persist at appreciable levels even after the removal of the antibiotic. This suggests that many resistant strains can evolve to be both resistant and have high fitness in absence of drug. To explore the conditions under which high fitness-resistant strains evolve and the genetic changes responsible, we used a combination of experimental evolution and whole genome sequencing to track the acquisition of ciprofloxacin-resistance in the opportunistic pathogen *Pseudomonas aeruginosa* under constant and fluctuating drug delivery patterns. We found that high fitness-resistant strains evolved readily under fluctuating but not constant drug

conditions and that their evolution was underlain by a trade-off between resistance and fitness. Whole genome sequencing of evolved isolates revealed resistance is gained through mutations in known resistance genes, and that second-site mutations generally compensated for costs associated with resistance via lab-adaptive changes. A broader spectrum of resistance mutations accrued in the fluctuating treatment, as selection in the variable environment favoured the evolution of a generalist that is both resistant and fit. Our results suggest that current drug therapies involving intermittent administration of antibiotics are contributing to the maintenance of antibiotic resistance at high levels worldwide.

3.1 Introduction

Comparative and epidemiological data suggest that the removal of an antibiotic from widespread use does not always lead to increased drug susceptibility in the population (Enne et al. 2001; Arason et al. 2002; Sundqvist et al. 2010). Indeed, the rate at which resistance declines is often much slower than expected based on measured costs of resistance (Andersson 2003; Andersson and Hughes 2010; Enne 2010) and resistant strains often persist at appreciable levels even in the absence of drug. This result is unexpected because many resistance mutations are energetically costly (Melnyk and Kassen 2015): an antibiotic-sensitive genotype not paying a cost of resistance should therefore rapidly displace resistant genotypes under permissive (no drug) conditions (Springer et al. 2001; Holberger and Hayes 2009; Stickland et al. 2010). The effectiveness of antibiotics is thus being undermined not only by the emergence of drug resistance but also by the unexpectedly long-term persistence of resistance in the absence of drug selection.

Managing the arsenal of useful antibiotics thus depends crucially on understanding how and why drug resistance is so persistent. We hypothesize that standard antibiotic therapies generate persistent drug resistance by selecting for the evolution of cost-free resistant strains. Drug delivery at the level of individual patients is best characterized as a series of pulses involving periods of high drug concentration immediately following administration or ingestion followed by periods of low drug concentration once the drug has been metabolized and removed from the body. Repeated drug dosing therefore generates a regular cycle of strong antibiotic selection followed by periods of relaxed selection where drug concentrations are greatly reduced or absent, a form of fluctuating selection. Fluctuating selection selects for broadly adapted generalists (Kassen and Bell 1998; Turner and Elena 2000; Kassen 2002; Buckling et al. 2006) that in this context are both drug resistant and maintain high fitness under permissive conditions. Such cost-free resistant genotypes are, we suspect, major contributors to persistent drug resistance.

The genetic mechanisms responsible for persistence are often attributed either to genetic co-selection due to linkage between the resistance gene(s) and selected loci (Borrell et al. 2013) or to compensatory mutations that alleviate the cost of resistance without compromising resistance itself (Björkman et al. 2000; Levin et al. 2000; Björkhom et al. 2001; Nagaev et al. 2001; Gagneux et al. 2006; Comas et al. 2012). However it is also possible that occasional 'no-cost' mutations, those that confer resistance but do not incur a large fitness cost under permissive conditions, are selected (Böttger et al. 1998; Gillespie et al. 2002; Pym et al. 2002; Sander et al. 2002; Ramadhan et al. 2005; Kassen and Bataillon 2006). The relative importance of these mechanisms to persistence remains unknown.

Here we test the hypothesis that fluctuating selection leads to the emergence of cost-free resistance genotypes by allowing initially isogenic populations of the opportunistic

human pathogen *Pseudomonas aeruginosa* to evolve under either persistent or fluctuating selection imposed by the commonly used fluoroquinolone antibiotic, ciprofloxacin. We then use whole-genome sequencing of the evolved populations to identify selected mutations and distinguish among the genetic mechanisms putatively responsible for cost-free resistance.

Our focus on *P. aeruginosa* stems from its role as a major cause of hospital infections and the pathogen most commonly associated with mortality in cystic fibrosis (CF) patients (U.K. Cystic Fibrosis Trust Antibiotic Working Group 2009; Canadian Cystic Fibrosis Foundation 2011). *P. aeruginosa* is known to maintain a high level of intrinsic resistance (Hancock and Speert 2000) and treatment with fluoroquinolones has been shown to lead to the rapid emergence of high levels of resistance to this class of drug (Wu et al. 1999). Moreover, the treatment of lung infections in CF patients with oral fluoroquinolone antibiotics is often periodic (also termed "intermittent administration"; U.K. Cystic Fibrosis Trust Antibiotic Working Group, 2009; Høiby et al. 2010), especially during acute exacerbations where lung function is compromised (Sordé et al. 2011). Standard treatment of exacerbations with ciprofloxacin typically lasts around 14 days (U.K. Cystic Fibrosis Trust Antibiotic Working Group, 2009; Bhatt 2013; da Silva Filho et al. 2013), and sometimes shorter to improve patient compliance and quality of life, especially for younger patients (Sordé et al. 2011). During periods of treatment bacterial levels decrease within the lung but are not eradicated (Fricks-Lima et al. 2011), implying that the evolution of high fitness resistant genotypes characteristic of compensatory evolution or the selection of cost-free mutants may be common. Our experiment is designed to capture many of the most salient features of treatment of *P. aeruginosa* infections. In particular, we have chosen drug concentration levels that mimic those seen in the sputum of CF patients undergoing

fluoroquinolone treatment (Pedersen et al. 1987) and have limited the length of our study to approximately the duration of a typical exacerbation treatment.

3.2 Results and Discussion

We followed the evolution of resistance and fitness in eight replicate populations of *P. aeruginosa*, serially transferred in LB in each of two environments: supplemented with daily (constant treatment) or intermittent (fluctuating treatment; see methods) doses of ciprofloxacin, for a total of approximately 50 generations of antibiotic selection. At the end of the experiment we isolated a single, random colony from each evolved population and assayed (1) minimum inhibitory concentration (MIC), the concentration of ciprofloxacin that inhibits growth, (2) growth rates in both the presence and absence of ciprofloxacin, and (3) relative fitness in competition with the sensitive ancestral strain under permissive treatments. Whole-genome sequencing of evolved isolates permitted the identification of the genetic targets of selection.

3.2.1 Fluctuating selection leads to the evolution of cost-free resistant genotypes

Selection was effective at generating ciprofloxacin resistance under both constant and fluctuating treatments, although isolates from the constant treatment had significantly higher MICs than those from fluctuating treatments (Fig. 3.1A, $W=54.5$, $p<0.01$). Evolved isolates from the constant treatment grew slower under both treatments (Fig. 3.1B, ciprofloxacin present: $t_{14}=-3.07$, $p=0.010$; Fig. 3.1C, ciprofloxacin absent: $t_{14}=-3.97$, $p<0.01$) and had lower

fitness relative to the ancestor (Fig. 3.1D, mean =0.833 +/- 0.0748 se; t_7=-2.23, p=0.06) than those from the fluctuating treatment (mean=0.963 +/- 0.0628 se, t_6=-0.587, p=0.580). These results point to the existence of a trade-off between the level of resistance and fitness measures: high levels of resistance evolving under constant antibiotic selection come at the cost of slower growth rates and lower fitness. These results also provide strong support for the hypothesis that the fluctuating drug-no drug treatments typically experienced by patients on standard antibiotic therapy selects for the evolution of drug resistant strains that maintain high fitness in the absence of antibiotic.

3.2.2 Genomics of adaptation

Whole-genome sequencing of the evolved isolates revealed between 2-5 mutations per isolate (mean=3.13 +/- 0.215se) using the Illumina MiSeq platform, which gave a median coverage of ~38-fold per genotype (mean=37.6; range 28.2-49.4) using 250-bp paired-end reads (Genome Quebec). The majority of mutations (n=33) were SNPs, but 2 insertions and 12 deletions were also uncovered (Table B.1 lists all mutations and their predicted functional consequences). Together, there were 31 unique changes affecting a total of 14 genes. No large insertions or deletions were found. Not included in the above calculations was a single isolate from the fluctuating treatment that bore a lesion in *mutS* and had 32 mutations, suggesting it is a mutator strain. Mutators have been shown to gain a fitness advantage when grown under antibiotic selective pressure *in vivo* (Meinike Jorgensen et al. 2013) and are often observed in clinical settings within the lungs of CF patients (Oliver et al. 2000; Oliver et al. 2004; Marvig et al. 2013; Marvig et al. 2014). However inferences about the genomic

targets of selection are complicated by elevated mutation rates so we do not consider this isolate further.

The mean number of mutations fixed under fluctuating selection (mean = 3.57 +/- 0.164 se) was marginally significantly higher than under constant selection (mean= 2.75 +/- 0.345 se; t_{14}=-2.04, p=0.074) although the fixation rate was significantly higher in the constant treatment (mean = 0.055 mutations/generation) compared to the fluctuating treatment (0.0357 mutations/generation; t_{14}=3.91, p<0.01). This apparently contradictory result comes about because we equalized the number of generations of antibiotic selection between the two treatments at ~50 generations, meaning that the fluctuating treatment necessarily evolved for twice as long as the constant treatment. Thus persistent directional selection imposed by constant ciprofloxacin causes faster rates of genomic evolution.

3.2.3 Genetic targets of resistance

Fluoroquinolone resistance occurs via two main mechanisms (Jacoby 2005; Ruiz 2003); (1) mutations to multidrug efflux pumps (MexCD-OprJ, MexAB-OprM) and their regulators (*nfxB*, *mexR*) that act to decrease intracellular fluoroquinolone concentrations (Poole 2005), and, (2) mutations that prevent fluoroquinolones from binding to DNA modifying subunits of DNA gyrases (*gyrA*, *gyrB*) or topoisomerases (*parC*, *parE*) (Yoshida et al. 1990a,b; Barnard and Maxwell 2001). We found that all isolates carried mutations in the efflux pump regulator *nfxB* and, with the exception of isolate f2, had a second resistance mutation in either *gyrA* or *gyrB*, suggesting that mutations in both types of targets are usually required to confer high levels of resistance (Fig. 3.2). This result agrees with previous work showing that combinations of mutations of these two types of targets lead to additive effects

on resistance (Bruchman et al. 2013). It is notable that parallel evolution at the nucleotide level within *nfxB* was higher in the constant than the fluctuating treatment: 7/8 evolved isolates from the constant treatment shared the same mutation, at amino acid position 14, while 5 distinct mutations at this gene were found in isolates from the fluctuating treatment. This result suggests that the range of genetic routes to resistance depends on the ecology of drug delivery, with more genetic solutions being available in more variable environments. Mutations in other known fluorquinolone resistance genes occurred more sporadically: isolate f4 harboured a *mexC* mutation and isolates c6 and f6 both had mutations in *orfN*, a gene encoding a predicted glycosyl transferase that has previously been suggested to confer increased resistance to ciprofloxacin (Wong et al. 2012). Thus resistance to ciprofloxacin can be conferred by a range of genetic targets in addition to the normal suite of efflux pump and gyrase/topoisomerase targets.

3.2.4 Adaptive second-site mutations are common

Additional mutations in genetic targets not obviously related to fluoroquinolone resistance, henceforth termed second-site mutations, were observed in nearly all evolved isolates except c1, c4, and f5 (Fig. 3.2). The fixation of a single second-site mutation in either *nfxB-gyrA* or *nfxB-gyrB* genetic backgrounds significantly improves fitness under permissive treatments, producing an average fitness effect (calculated as the difference in fitness of the triple mutant over the double mutant scaled by the fitness of the double mutant) of 0.837 ± 0.238 se (Table 3.1: $t_7 = 3.53$, $p < 0.01$). The main target of second-site mutations appears to involve a shift from a motile, planktonic lifestyle to a non-motile, biofilm-forming state, as evidenced by mutations in *morA*, *wspA*, and *wspF*, all genes that are known to affect

motility and biofilm formation via cyclic-di-GMP signaling (Wong et al. 2012; Meissner et al. 2007; Hickman et al. 2005; Wolfe and Visick 2008). Loss of motility is likely to be adaptive in our experiment because cultures were maintained with constant agitation, meaning the maintenance and assembly of the complex machinery associated with motility is unnecessary. Other genetic targets included heme biosynthesis (*hemN*) and type IV pili formation (*pilC*), both of which harboured frame-shift mutations towards the beginning of the gene that likely produce a truncated protein and so are expected to result in loss of function. Taken together, these results suggest that the fitness benefit associated with second-site mutations results primarily from mutations that prevent energy being expended on the expression and maintenance of costly functions not necessary for growth under laboratory conditions.

3.2.5 The genetics of cost-free resistance

It is tempting to interpret the evolution of cost-free resistance under fluctuating treatments as being due to the substitution of second-site mutations that improve fitness but do not compromise resistance. On the face of it, the observation that more mutations were fixed in isolates from the fluctuating treatment than the constant treatment is consistent with this interpretation. However, three observations argue against this interpretation.

First, while nearly all isolates contained at least two known resistance mutations, in *nfxB* and either *gyrA* or *gyrB*, many of the isolates from the fluctuating treatment contained additional mutations in known fluoroquinolone resistance targets. Isolate f5, for example, contains three resistance mutations, in *nfxB*, *gyrA*, and *gyrB*. Other examples include isolate f4 with mutations in *gyrA*, *nfxB* and also in *mexC* (Hooper, 2001) and isolate f6 with

additional mutations in both *orfN* (Wong et al. 2012) and *oprJ* mutations (Jalal et al. 2000; Hooper, 2001). Thus at least some of the additional mutations selected in the fluctuating treatment are linked to increased resistance rather than fitness under permissive conditions. Second, the fixation of second-site mutations is not restricted to the fluctuating treatment. In particular, isolates from the constant treatment had mutations in *morA, wpbM*, *pilC,* and *hemN*, all genes that have no obvious connection to resistance. It is further notable that *morA* mutations, some of which have previously been shown to be associated with increased aggregation and biofilm formation (for example, L1155Q; Wong et al. 2012), were fixed in both treatments, making it difficult to see how these mutants could be specifically associated with fitness improvements under the permissive conditions available in the fluctuating treatment. Finally, second-site mutations from the constant environment are often beneficial. With the exception of the *pilC* mutation, which appears to be neutral with respect to fitness and so presumably hitchhiked to high frequency alongside a resistance mutation, the fitness of genotypes containing second-site mutations in the constant treatment is significantly greater than that of two comparable genotypes (c1 and c4) with shared or very similar resistance mutations but lacking second-site mutations (Table 3.1). This is especially notable with isolate c5, which had mutations in *nfxB*, *gyrB*, and *hemN*, and was both highly resistant and fitter than the sensitive ancestor under permissive treatments despite having evolved under constant drug conditions. Evidently, second-site mutations can alleviate costs of resistance incurred in the presence of drug regardless of mode of treatment. Lending support to this interpretation, second-site mutations were nearly as common in the constant environment as they were in the fluctuating environment: 5/8 isolates in the constant treatment compared to 6/7 isolates in the fluctuating treatment, a result that is not significantly different (χ^2=0.184, df=1, p=0.668). Taken together, these results suggest that

cost-free resistance cannot be driven exclusively by the fixation of second-site compensatory mutations.

Rather, we suspect that the evolution of cost-free resistance is attributable to the selection of low-cost resistance alleles in the fluctuating environment. While we do not have direct estimates of the MICs or fitness values associated with specific mutations, two indirect pieces of evidence are consistent with this interpretation. First, the most commonly mutated gene in our experiment, *nfxB*, fixed the same L14P allele in 7/8 strains in the constant environment but was found in only 1/7 strains in the fluctuating environment, a statistically significant difference (χ^2=5.37, df=1, p=0.020) and suggests that the L14P allele is much more beneficial under constant conditions than under fluctuating conditions. We suspect the benefit of this allele stems from the high level of resistance it confers, despite it being very costly. Future work will test this prediction directly by measuring the MIC and fitness value of the mutation *nfxB* L14P. Second, there appears to be a wider range of novel combinations of resistance alleles evolving under fluctuating conditions than constant conditions, with at least one uncommon *gyrA* mutation in strain f2 (A615E), the fixation of mutations in *mexC* (f4) and *oprJ* (f6) in addition to those in *nfxB* and *gyrA*, and the fixation of an *nfxB-gyrA-gyrB* genotype in strain f5. None of these combinations of mutations were observed in constant conditions. We suspect that the levels of resistance conferred by these alleles is substantially less than those selected under constant conditions and, moreover, that the costs associated with these mutations are also less. Thus maintaining a sufficient level of resistance in the fluctuating environment requires the substitution of multiple weakly resistant alleles that, together, do not incur large costs of resistance.

3.2.6 A model for the evolution of persistent drug resistance

While the evolution of resistance is widely and justifiably recognized as a costly trait, we have shown how these costs can be readily circumvented by the selection of resistant mutants that pay little cost under fluctuating drug treatment. The range of low-cost resistant mutations may be quite limited, as it appears to be constrained by an underlying trade-off between resistance and fitness under permissive conditions. Nevertheless fluctuating conditions provide ample opportunity for the selection of novel combinations of mutations conferring low-cost resistance. The variable selective conditions associated with fluctuating selection no doubt helps, as a genotype harbouring even a modest level of resistance may be able to survive long enough to substitute additional resistance mutations or second site mutations that improve fitness under permissive conditions. Similar heterogeneity in environmental conditions is not available under constant drug selection, so the range of genetic routes to adaptation is more constrained.

That said it is notable that constant drug selection can lead to the occasional evolution of genotypes that are both resistant and maintain fitness in the absence of drug. High costs of resistance can therefore be alleviated by mutations that afford very general fitness improvements to the prevailing treatments of growth, although the trade-off between resistance and fitness ensures that such genotypes are probably not common. Nevertheless, in large populations, competition among distinct resistance mutations may lead to the evolution of what we might call ‘high resistance, cost-reduced’ genotypes. These genotypes are likely to be less fit in the absence of drug than those that evolve under fluctuating treatments but they are likely to be more resistant, and so may be an under-appreciated source of antibiotic persistence.

Our results present an admittedly bleak picture for dealing with persistent drug resistance. To the extent that our experiment captures the essential ecology of drug delivery associated with many current therapies such as intermittent administration of oral antibiotics during exacerbations within CF patients, our results suggest that these therapies are likely selecting directly for cost-free resistant genotypes. Moreover, our results also suggest that occasional low-cost resistant genotypes may occasionally evolve even under more persistent drug administration regimes, making the management of antibiotic persistence even more difficult. Taken together, these results suggest the most effective strategy for dealing with persistence is to ensure that either resistance does not evolve in the first place, perhaps through treatment with multidrug cocktails as is normally practiced in HIV therapy, or to exploit the trade-off between resistance and fitness under permissive conditions by ensuring that when resistance evolves, it is most likely to do so through mutations that incur a high fitness cost.

3.3 Methods

3.3.1 Experimental evolution

A single colony of *P. aeruginosa* isolate PA14 was grown overnight in Lysogeny broth (LB) broth (bacto-tryptone 10g/L, NaCl 3 g/L, yeast extract 5 g/L). Sixteen populations were founded from this progenitor by adding 20 uL of overnight culture to 1.5 mL of fresh medium (media described below). An aliquot of progenitor was frozen at -80^{o}C in glycerol. Populations were grown in an orbital shaker (150 rpm) at 37^{o}C for 24 hours in 24-well plates

(Corning, NY, USA). After 24 hours each population was serially propagated by transferring 20 uL of overnight culture to 1.5 mL of fresh medium. Overnight cultures were frozen at -80^{o}C in glycerol (%vol). Overnight cultures were frozen every eight transfers, such that approximately 50 generations of adaptation occurred each cycle.

Two selection treatments were used, consisting of LB broth with or without antibiotic. For all the antibiotic treatments a concentration of 0.8 ug/mL ciprofloxacin was used, because it decreased growth of the sensitive PA14 ancestor to 20% of full growth in LB broth. A concentration of ciprofloxacin that completely inhibited growth was not used because we needed bacterial growth to continue our selection experiment. The two treatments differed in the amount of time the populations were allowed to grow in the absence of the antibiotic. Populations were grown in the ciprofloxacin via two different temporal treatments: a constant treatment (c) where populations were exposed to ciprofloxacin for the entirety of the 50 generations, and a fluctuating treatment (f). The fluctuating treatment involved populations growing for two cycles (48 h) of growth in the presence of the antibiotic followed by two cycles of growth in the absence of the antibiotic, and then repeating each sequence for a total of 16 growth cycles, and a total of 50 generations of growth in the presence of ciprofloxacin. Comparable experimental evolution studies examining the evolution of antibiotic resistance used similar time scales (24 h alternation; Kim et al. 2014: total length of ~50 generations; Wong et al. 2012).

3.3.2 Phenotypic analyses

For each evolved population a single isolate of the most common type was obtained from each end-point evolved population. For each evolved isolate, the level of resistance was

assayed as the minimum inhibitory concentration (MIC) of ciprofloxacin. Overnight cultures of each evolved isolate were grown in LB broth, of which 10 uL was diluted into 10 mL LB broth and 100 uL of this dilution was used to inoculate 96-well plates with varying concentrations of ciprofloxacin. For each evolved isolate we assayed growth at 0x, 1x, 2x, 4x, 8x, 16x, 32x, 64x, 128x, 256x, and 512x the ancestral MIC. Log-transformed MICs were used for all statistical analyses.

Relative maximum growth rate of the evolved isolates in the presence and the absence of ciprofloxacin were measured as the maximum growth rate of each isolate over a 24-hour period relative to the ancestral growth rate of PA14 in LB broth. For each assay 20 uL of overnight culture was added to 180 uL of LB broth and OD600 was measured by spectrophotometry every 90 minutes. Growth rate during the exponential phase was then estimated using Gen5 software (Bio Tek Instruments Inc., Winooski, VT), and relative growth rate was taken as the mean growth rate of 3 replicates divided by the mean growth rate of the ancestral PA14 genotype grown on the same assay plate. Log-transformed mean relative growth rate was used for all statistical analyses (Houle et al. 2011).

Relative competitive fitness of each evolved isolate was assayed using a competitive fitness assay against a lacZ-marked ancestral isolate. Independent assays verified that the lacZ-marked strain did not bear a fitness cost in competitions with unmarked PA14. Both competitors were grown overnight in LB broth, the competition medium. At time 0, 10 ul of the marked ancestor and 10ul the evolved isolate were inoculated into 1.5mL of fresh medium in a 24-well plate and an aliquot of the mix was frozen at -80^{o}C in glycerol. Following 24 hours of growth at 37^{o}C at 150 rpm, a final aliquot was frozen in -80^{o}C in glycerol. Serial dilutions of initial and final aliquots were grown on solid minimal media + X-gal, allowing us to determine the numbers of blue (ancestral) and white (evolved)

individuals at the beginning and end of the competition. The selection coefficient, *s*, was calculated as:

$$(\ln(\frac{whitefinal}{whiteinitial}) - \ln(\frac{bluefinal}{blueinitial}))/(\#\ \text{of generations}) \quad (3.1)$$

Relative fitness, *w*, was then calculated as 1+*s*, where the units for *w* and *s* are in per generation.

All statistical analyses were performed in R (R Core Team, 2013).

3.3.3 Whole-genome sequencing and analysis

For whole-genome sequencing, each evolved isolate used in the above analysis were grown overnight in LB broth, and genomic DNA was extracted using the Promega Wizard Genomic DNA Purification kit. Genome Quebec Innovation Centre performed 250-bp paired-end Illumina sequencing using the Mi-Seq platform. Mean coverage across all 16 genotypes was 38-fold (mean= 37.7; range 28.2-49.4) at a mean quality score of 32. The sequence data were aligned to PA14 reference genome number NC_008463.1, and mutations were called and annotated using a custom computational pipeline (modified from (Dettman et al. 2012), and then all mutation alignments were checked visually using SamTools (ver. 0.1.19).

A subset of mutations was verified by Sanger sequencing of polymerase chain reaction (PCR) amplicons; for 20 mutations (out of 47 mutations identified in 15 evolved isolates), we amplified a 500-700 base pair (bp) PCR product containing the putative

mutation, and directly sequenced the PCR products (Genome Quebec, Montreal). All 20 mutations that we examined were successfully verified. Gene location and function were found using the Pseudomonas genome database (Winsor et al. 2011).

3.3.4 Annotation of gene function

Annotation of gene function was done using the Pseudocap function as listed on the Pseudomonas genome database (Winsor et al. 2011). We supplemented the "antibiotic resistance and susceptibility " class with additional genes that were known to be involved with fluoroquionlone resistance in *P. aeruginosa*. These included (1) subunits of the targets of ciprofloxacin, DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE;* Barnard and Maxwell 2001; Yoshida et al 1990a; Yoshida et al. 1990b), and (2) the efflux pumps MexAB-OprM and it's regulator *mexR*, and MexCD-OprJ and it's regulator *nfxB* (Poole 2005). Additionally, genes with crosshatched notation for the functional class "antibiotic resistance and susceptibility" are genes that have been hypothesized to be involved with fluoroquionlone resistance (*orfN*; Wong et al. 2012, this study).

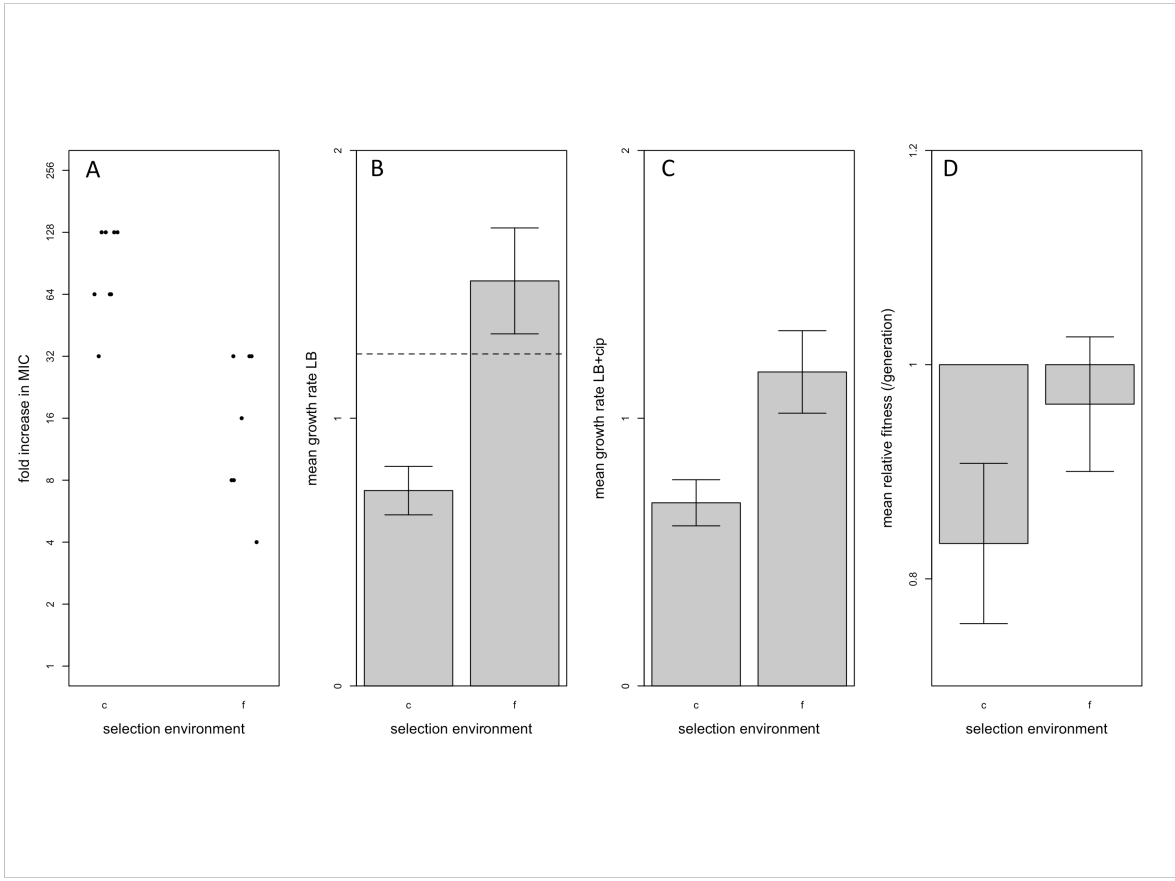
A
fold increase in MIC
1
2
4
8
16
32
64
128
256
c
f
selection environment
B
mean growth rate LB
0
1
2
c
f
selection environment
C
mean growth rate LB+cip
0
1
2
c
f
selection environment
D
mean relative fitness (/generation)
0.8
1
1.2
c
f
selection environment

Figure 3.1. Adaptive responses as measured by (A) minimum inhibitory concentration (MIC) of ciprofloxacin, (B) mean growth rate in the absence, and (C) presence of ciprofloxacin, and (D) mean relative fitness of evolved isolates. All error bars represent +/- 1SE. MIC was calculated as the median of two trials. Mean growth rate was calculated from three replicate measures for each experimentally evolved isolate. Mean relative fitness was measured in the absence of antibiotic via direct competitions with a lacZ marked ancestral isolate (PA14) using three replicate measures from each experimentally evolved isolate. A relative fitness of less than one indicates a cost of resistance in the absence of the antibiotic.

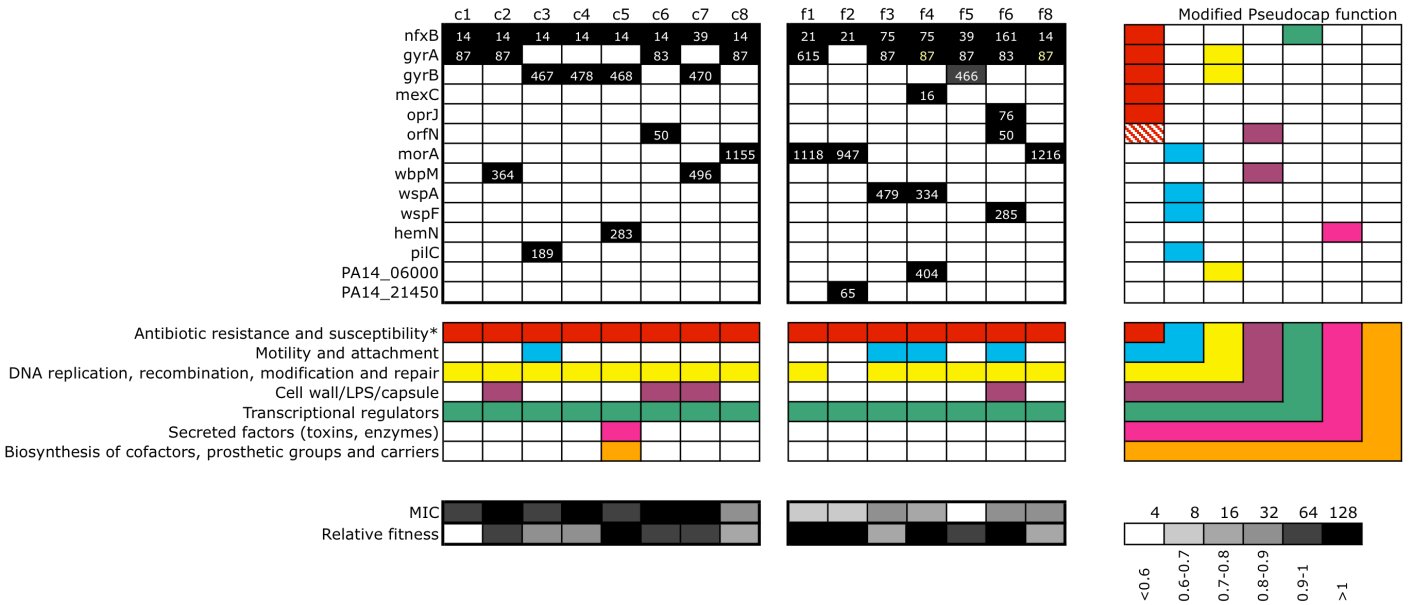

Figure 3.2. Genes containing mutations (n=14) after selection in constant or fluctuating ciprofloxacin treatment. The black squares in the large matrix denote genes that had mutational changes and the numbers within the boxes represent the codon number where the change occurred. Different coloured numbers represent different amino acid changes. The coloured squares in the smaller matrices denote PseudoCap (Winsor et al. 2011) functions of the genes, and the heat map for MIC and fitness represent the phenotypic analyses for this study, with darker colours representing higher MIC and greater fitness, respectively.

Table 3.1. Fitness effect of second-site mutations for all evolved isolates in both selection treatments. The bold fitness effect at the bottom of the table is the mean second-site fitness effect +/- standard error.

Isolate (mutations)	Relative fitness	Fitness effect
c1 (nfxB + gyrA) [1]	0.364	
c2 (nfxB + gyrA + wbpM)	0.944	1.591
c6 (nfxB + gyrA + orfN)	0.936	1.569
c8 (nfxB + gyrA + morA)	0.724	0.988
f1 (nfxB + gyrA + morA)	1.014	1.781
f3 (nfxB + gyrA + wspA)	0.760	0.521
f8 (nfxB + gyrA + morA)	0.714	0.959
c4 (nfxB + gyrB) [1]	0.895	
c3 (nfxB + gyrB + pilC)	0.831	-0.071
c5 (nfxB + gyrB + hemN)	1.053	0.176
c7 (nfxB + gyrB + wbpM)	0.914	0.021
		0.837 (+/- 0.237)

[1] isolates used as the double-mutant for calculation of fitness effect

Chapter 4

Lack of epistasis between ciprofloxacin-resistance mutations in *Pseudomonas aeruginosa*

This chapter is reproduced from:

Melnyk, A. H., N. McCloskey, A. Hinz and R. Kassen. (in prep.) "Lack of epistasis between ciprofloxacin-resistance mutations in *Pseudomonas aeruginosa*."

Collaborator contributions:

N. McCloskey and A. Hinz made the allelic constructs and assessed their relative fitness and MIC.

Abstract

Epistatic interactions have the potential to play a crucial role in the spread of antibiotic resistance in pathogen populations by affecting the fitness cost associated with resistance mutations in antibiotic-free environments. We investigated the fitness costs of ciprofloxacin resistance mutations both singly and in combination, and estimated the epistatic effects of 12 pairs of ciprofloxacin-resistance mutations that arose during a previous evolution experiment using the pathogenic bacterium *Pseudomonas aeruginosa*.

We show that mutations associated with different ciprofloxacin resistance mechanisms have different costs; mutations that result in the over-expression of efflux pumps are costly, whereas mutations in the target of ciprofloxacin, DNA gyrase, are nearly cost-free. Surprisingly, we only detected one case of antagonistic epistasis, whereby the double resistant mutant was less costly than expected. These results suggest that epistatic interactions between antibiotic resistance mutations that arise together in an evolved genome may not be as common as the literature would have us believe.

4.1 Introduction

The evolution of antibiotic resistance in bacteria often involves the substitution of multiple resistance mutations (Anderson 2005; Jacoby 2005; Lindgren et al. 2005; Weinreich et al. 2006; Marcusson et al. 2009; Melnyk et al. in prep). As resistance mutations co-occur, epistatic interactions, that is the phenotypic effect of one mutation being dependent on another mutation present elsewhere in the genome, could potentially play a key role in the evolution of resistance. Specifically, epistasis can play a role in determining the fitness cost associated with antibiotic resistance. Individual resistance mutations often incur a fitness cost in the absence of antibiotic (Andersson 2003, 2006; Melnyk et al. 2015) and this cost plays a key role in determining the spread of resistance mutations in pathogenic populations (Andersson & Hughes 2010). The nature of epistatic interactions between resistance mutations will determine the cost of carrying multiple resistance mutations, and therefore the strength of selection for or against resistance. For example, in a scenario where resistance mutations are costly, if there is antagonistic

epistasis between two resistance mutations, the cost of carrying both mutations will be relatively less than expected, and epistasis will promote the evolution of antibiotic resistance (Figure 4.1). Conversely, if synergistic epistasis occurs between two costly resistance mutations, the cost of carrying both mutations will be greater than expected, and epistasis will constrain their spread (Figure 4.1). Such knowledge can be used to understand and predict what type of resistance mutations are likely to be segregating in microbial populations (Gagneux et al. 2006).

Table 4.1. Glossary

Epistasis: When the fitness effect of a mutation is modulated by its interactions with other genes or mutations in the genome. **Antagonistic epistasis:** When double mutant fitness is less than expected based on single mutant fitness values (i.e. fitness of double mutant is closer to W=1 than predicted based on W_{Ab}*W_{aB}; see upper left and bottom right panel Figure 4.1). **Synergistic epistasis:** When double mutant fitness is greater than expected based on single mutant fitness values (i.e. fitness of double mutant is farther from W=1 than predicted based on W_{Ab}*W_{aB}; see bottom left and upper right panel of Figure 4.1).

A small number of studies have tested for epistasis in terms of fitness costs between resistance mutations (Reynolds 2000; Rozen et al. 2007; Trindade et al. 2009; Ward et al. 2009; Borrell & Gagneux 2011; Hall et al. 2011; Borrell et al. 2013; Schenk

et al. 2013) and have consistently found evidence of a bias towards antagonistic epistasis (combinations of resistance mutations are less costly than expected). Several studies investigating epistatic interactions among pairs of mutations that confer resistance to two different drugs have shown that in some combinations, previously costly alleles become beneficial (Ward et al. 2009; Trinidade et al. 2009; Borrell et al. 2013). Other studies have focused on the epistatic interactions among mutations that confer resistance to a single drug (Gagneux et al. 2006; Rozen et al. 2007; Hall et al 2011; Borrell & Gagneux 2011; Borrell et al. 2013). In these studies it was found that antagonistic epistasis was prevalent among pairs of antibiotic resistance mutations. These results suggest that epistasis between intragenic resistance mutations may be a key determinant of the cost of antibiotic resistance and compensatory adaptation in pathogen populations. However none of these studies used mutations that arose experimentally in response to sustained antibiotic selection pressure. Additionally, for the subset of studies that examined resistance mutations to the same drug, in general they examined epistasis between mutations within the same gene.

In this study we examined the fitness effects, both alone and in combination, of antibiotic resistance mutations that arose during a previous selection experiment that adapted the pathogenic bacterium *P. aeruginosa* to the fluoroquinolone antibiotic ciprofloxacin for 50 generations. We wanted to know whether different resistance mechanisms incurred variable fitness costs. Fluoroquinolone resistance occurs via two main mechanisms (Ruiz 2003; Jacoby 2005); (1) mutations in the regulators of multidrug efflux pumps (*nfxB, mexR*) that result in over-expression of these pumps and decreased intracellular fluoroquinolone concentrations (Poole 2005), and, (2) mutations in the target

of fluoroquinolones, DNA gyrases (*gyrA*, *gyrB*) or topoisomerases (*parC*, *parE*; Yoshida et al 1990a, b; Barnard & Maxwell, 2001). To investigate whether different resistance mechanisms had different fitness costs, we contrasted the fitness effects of single mutations that arose in the efflux pump regulator *nfxB*, and compared that to the fitness effects of mutations in the DNA gyrase genes *gyrA* and *gyrB*. In our evolution experiment multiple resistance mutations were almost always found in combination within a single isolate, usually combining the two resistance mechanisms. We considered this a striking result, and thus measured the fitness effects of these mutations in combination, and quantified their epistatic interactions.

In *P. aeruginosa*, to our knowledge only epistatic fitness interactions between rifampicin resistance mutations have been studied (Ward et al. 2009; Maclean et al. 2010; Hall et al. 2011). Resistance to rifampicin is through mutations in the drug target, *rpoB*, and thus all cases of epistasis studied have been intragenic. With rifampicin resistance, epistasis is pervasive and tends to be antagonistic. Sign epistasis is also common, whereby pairs of deleterious mutations partially or completely compensate for each other's costs. With respect to ciprofloxacin, epistasis between different resistance mutations has been studied in *Streptococcus pneumoniae* (Rozen et al. 2007). Naturally occurring *parC*, *parE* and *gyrA* mutations were studied, and it was found that most mutations were slightly costly alone, but again exhibited antagonistic epistasis such that the addition of a second mutation did not increase the fitness cost imposed by the single mutation. Note that these mutations were all within the targets of ciprofloxacin, and none were within efflux pump regulators. A single study has examined ciprofloxacin resistance conferred by both target alterations and efflux pump regulator knockouts (Bruchman et

al. 2013), however, they only measured increased in resistance, measured as increase in minimum inhibitory concentration of ciprofloxacin, and did not examine the fitness costs of resistance. Taken together these previous studies would lead us to hypothesize that there will be antagonistic epistasis between the resistance mutations examined here.

In this study, we show that the fitness costs of individual ciprofloxacin-resistance mutations are dependent upon the resistance mechanisms targeted. Mutations in an efflux pump regulator (*nfxB*) were costly, whereas mutations in the ciprofloxacin target DNA gyrase (*gyrA* and *gyrB*) were similar in fitness to the wild-type. We further show that there are minimal epistatic interactions between pairs of ciprofloxacin-resistant mutations, which challenge the results of previous studies that generally showed strong, significant antagonistic epistatic interactions. However, previous studies assayed the potential for epistatic interactions between any resistance mutations, whereas our study measures realized epistatic interactions between pairs of resistance mutations that are the result of selection to ciprofloxacin.

4.2 Materials and Methods

4.2.1 Bacterial Strains

The bacterial genotypes and mutations used in this study were isolated from a previous selection experiment designed to explore the dynamics of adaptation to ciprofloxacin under different temporal drug delivery treatments (for details see Melnyk et al. in prep). Briefly, we followed the evolution of resistance and fitness in eight replicate

populations of *P. aeruginosa*, serially transferred in Lysogeny broth (LB) in each of two environments: supplemented with daily (constant treatment) or intermittent (fluctuating treatment) doses of ciprofloxacin. After a total of approximately 50 generations of antibiotic selection, we isolated a single, random colony from each evolved populations and sequenced their genomes. From these 16 isolates, 12 had pairs of mutations in known resistance conferring genes.

4.2.2 Allelic Constructs

The *gyrA*, *gyrB*, and *nfxB* mutations were introduced in single or double mutant combinations into the Pa14 chromosome using a previously described allelic exchange method (Rainey 1999, Bailey et al. 2014). Mutations and surrounding sequences were PCR amplified from chromosomal DNA isolated from the evolved bacteria and cloned into a derivative of pAH79, a suicide vector with ampicillin and tetracycline resistance genes and a *sacB* counter-selectable marker (Bailey et al. 2014). The plasmids were mobilized into Pa14 by tri-parental mating with the help of pRK2013. Chromosomal integration of the plasmid by homologous recombination was selected using LB agar supplemented with 100 μg/ml nitrofurantoin and 60 μg/ml tetracycline. A second recombination, resulting in loss of plasmid and marker-less allelic replacement, was selected by *sacB* counter-selection. Tetracycline-resistant clones were plated on LB agar containing 5% sucrose. Successful allelic replacements were distinguished from wild-type revertants by Sanger sequencing. Double mutants were constructed by introducing an additional mutation by the same procedure.

4.2.3 Measuring fitness

Relative competitive fitness of each construct was assayed using a competitive fitness assay against the lacZ-marked ancestor PA14. Independent assays verified that the lacZ-marked strain did not bear a fitness cost in competitions with unmarked PA14. Both competitors were grown overnight in LB broth, the competition medium. At time 0, 10 ul of the marked ancestor and 10ul the evolved isolate were inoculated into 1.5mL of fresh medium in a 24-well plate and an aliquot of the mix was frozen at -80^{o}C in glycerol. Following 24 hours of growth at 37^{o}C at 150 rpm, a final aliquot was frozen in -80^{o}C in glycerol. Serial dilutions of initial and final aliquots were grown on solid minimal media + X-gal, allowing us to determine the numbers of blue (ancestral) and white (evolved) individuals at the beginning and end of the competition. The selection coefficient, *s*, was calculated as:

$$(\ln(\frac{whitefinal}{whiteinitial}) - \ln(\frac{bluefinal}{blueinitial}))/(\#\ \text{of generations}) \tag{4.1}$$

Relative fitness, *w*, was then calculated as 1+*s*, where the units for *w* and *s* are in per generation.

4.2.4 Measuring epistasis

Fitness of double resistant constructs was used to calculate pair-wise epistasis assuming a multiplicative model. Pair-wise epistasis, ε, between locus A and B can be measured as follows (Kouyos et al. 2007). If W_{AB} is the fitness of the wild-type, W_{Ab}, W_{aB} are the fitnesses of each of the single mutants and W_{ab} that of the double mutant then:

$$\varepsilon = W_{AB}W_{ab} - W_{Ab}W_{aB} \tag{4.2}$$

A positive value of ε indicates that the double mutant fitness is higher than expected based on single mutant fitness values. Similarly, a negative value of ε indicates that double mutant fitness is lower than expected based on single mutant fitness values. To test whether epistasis values were significantly different from zero, we used the error propagation method described by Trindade and colleagues (2009). Briefly, error, σ_ε, of the value of ε is then estimated by:

$$\sigma_\varepsilon = \sqrt{(W_{ab}^2\,\sigma_{WAB}^2 + W_{AB}^2\,\sigma_{Wab}^2 + W_{aB}^2\,\sigma_{WAb}^2 + W_{Ab}^2\,\sigma_{WaB}^2)} \tag{4.3}$$

Whenever the value of $|\varepsilon|$ was within the range of $|\sigma_\varepsilon|$, we considered that alleles *a* and *b* did not show any significant epistasis.

Epistasis can also be estimated from an additive model:

$$\varepsilon_a = \alpha\, ab - (\alpha\, Ab + \alpha\, aB) \tag{4.4}$$

where $\alpha\, ab$ is the fitness effect of having alleles *a* and *b* (Phillips 2008; Trinidade et al. 2009). Fitness effect for a given allele was calculated as

$$\alpha\, Ab = W_{Ab} - W_{ab} \tag{4.5}$$

Under and additive model, we obtained the same qualitative results as with the multiplicative model, because the distribution of ε was extremely similar under both models (Kolmogorov-Smirnov test: D=0.333, P=0.536).

4.2.5 Measuring resistance

For each construct the level of resistance was assayed as the minimum inhibitory concentration (MIC) of ciprofloxacin. Overnight cultures of each evolved isolate were grown in LB broth, of which 10 uL was diluted into 10 mL LB broth and 100 uL of this dilution was used to inoculate 96-well plates with varying concentrations of ciprofloxacin. For each evolved isolate we assayed growth at 0x, 1x, 2x, 4x, 8x, 16x, 32x, 64x, 128x, and 256x the ancestral MIC. We performed 3 replicate wells for each construct at each concentration, and reported the median fold-increase in resistance. Log-transformed MICs were used for all statistical analyses.

All statistical analyses were performed using R vers. 3.1.1.

4.3 Results

To study the cost of single resistance alleles, and the degree of epistatic interactions amongst alleles that confer ciprofloxacin resistance in experimentally evolved populations of *P. aeruginosa*, we made allelic replacement constructs of single and double resistance mutations in combinations that arose in a previous selection experiment (Chapter 3). We surveyed 13 mutations in three known resistance conferring genes; *gyrA*, *gyrB* and *nfxB*. For each construct we assayed (1) ciprofloxacin resistance, measured as minimum inhibitory concentration (MIC), that is the concentration of ciprofloxacin that inhibits growth, and (2) relative fitness in competition with the sensitive strain under permissive (i.e. no antibiotic) conditions, to assess fitness costs of these mutations.

4.3.1 Fitness costs of individual resistance mutations

We determined the cost of resistance of each of the mutations in the absence of ciprofloxacin by measuring the relative fitness of each construct in competition with the wild-type ancestor. The relative competitive fitness values of all resistant constructs are provided in Table C.1. Figure 4.2 shows the mean fitness effect of each single mutation, grouped by gene. Individual resistance mutations tended to carry a fitness cost (mean fitness for all individual resistant mutants = 0.963, SE=0.021), although there was variation in fitness values, and 7 of 13 mutants did not exhibit a cost. There was a significant effect of gene on relative fitness (F=25.8, P<0.001), with *nfxB* (Figure 4.2A;

mean fitness = 0.863 SE=0.026) constructs being significantly less fit than *gyrB* constructs (Figure 4.2B; mean fitness = 1.003 SE=0.014) and *gyrA* constructs (Figure 4.2C; mean fitness = 1.012 SE=0.002). Additionally, *nfxB* constructs were significantly costly (t_3=-5.53, P<0.05), whereas *gyrA* and *gyrB* constructs on average were not.

4.3.2 Epistasis between ciprofloxacin-resistance mutations tends to be antagonistic

To study the fitness costs of double mutant constructs and measure epistatic interactions, we constructed all double mutant combinations of resistance alleles that arose within the same evolved isolate in a previous selection experiment (Melnyk et al in prep). These correspond to 7 *gyrA*+*nfxB* double mutants, 4 *gyrB*+*nfxB* double mutants and a single *gyrA*+*gyrB* double mutant. To measure epistasis, we estimated ε for each double mutant; this quantifies the deviation of double mutant fitness from that predicted by multiplying single mutant fitness values together (see Materials and Methods). Observed fitness values of double mutants were generally greater than expected fitness values (Figure 4.3). For a double mutant comprising two single mutations that are both costly, a positive value of ε represents antagonistic epistasis whereby the double mutant is less costly than expected. Epistasis was generally antagonistic, as demonstrated by the fact that the average value of ε (mean=0.052, SE=0.014) was significantly greater than zero (t_{11}=3.62, P<0.01; Figure 4.4), and the expected fitness of the double mutants was generally less than one. However, once we tested whether the values of ε were significantly different from zero using the error propagation method described by

Trinidade and colleagues (2009), only one pair of mutations exhibited significant antagonistic epistasis (Figure 4.5).

Previous studies (Trinidade et al. 2009; Hall et al. 2011) and studies in digital organisms (Wilke & Adami 2001) have shown that epistasis and the fitness effect of mutations can be correlated, such that mutations with larger effects are more epistatic. In our study there was a marginally significant negative correlation between the value of ε and the expected fitness of the double mutant (Figure 4.4; Pearson's correlation r=-0.520, *P*=0.083). Recall in our study that the fitness effects of single resistance mutations were generally negative (mean fitness for all resistant mutants = 0.963, SE=0.021), therefore the marginally significant negative correlation between ε and expected fitness is in agreement with previous findings that single mutations with larger fitness effects (i.e. more costly in this study) are more epistatic.

4.3.3 Lack of sign epistasis

Sign epistasis exists when a mutation is deleterious on some genetic backgrounds but beneficial on others. This form of epistasis, if common, may give rise to multiple fitness peaks on the fitness landscape, thereby constraining the evolution of resistance (Weinreich et al. 2005). In the context of antibiotic resistance, if sign epistasis is pervasive it will be much more difficult to move from resistant peaks back to sensitive peaks, even if antibiotic selection pressure is stopped. The most important example of sign epistasis in the context of antibiotic resistance evolution is when two individually costly resistance mutations, in combination, are beneficial, and thus also compensatory.

Sign epistasis has been shown between resistance mutations in several studies (Trinidade et al. 2009; Hall et al. 2011) Similarly, experimental evolution studies have shown that, within hundreds of generations, mutations that compensate for the cost of antibiotic resistance are more likely to occur (Schrag & Perrot 1996; Björkman et al. 2000; Levin et al. 2000; Gifford & MacLean 2013) than revertants (Maisnier-Patin et al. 2002). Despite the suggestion that sign epistasis might have an important role for the evolution of antibiotic resistance, our study found no evidence of sign epistasis between evolved resistance mutations.

4.3.4 Why are these resistance mutations found together?

The combinations of resistance mutations that we studied were selected together within a single isolate, and thus there should be some adaptive advantage to having both mutations. To see the selective advantage of the double constructs we examined the concurrent gains in resistance that are accompanied by the addition of a second resistance mutation. Singly, mutations increased the MIC of ciprofloxacin between 1-8 fold, with a median 4-fold increase (Figure 4.6). Double constructs had MICs that ranged from 8-64 fold increase, with a median of 16-fold increase (Figure 4.6), which is a significant increase over the single allele constructs (W=156, $P<0.0001$). There is no correlation between double construct MIC and it's respective cost of resistance (Pearson's correlation r=0.102, $P=0.752$). Thus double constructs are likely selected because they confer increased resistance. This result was not unexpected as combinations of mutations that conferred both inactivation of the efflux regulator gene, and drug target alteration,

have previously been found to increase ciprofloxacin-resistance to a greater extent than multiple mutations within a single resistance mechanism (Bruchman et al. 2013).

4.4 Discussion

We have shown that single ciprofloxacin-resistance mutations are generally costly, and that epistatic interactions are not prevalent in determining the cost of acquiring multiple ciprofloxacin-resistance mutations in *P. aeruginosa*.

4.4.1 Ciprofloxacin-resistant mutations are costly

We have focused explicitly on measuring the fitness costs of ciprofloxacin-resistance mutations that arose in a previous selection experiment. Single resistance mutations were the most costly in the efflux pump regulator *nfxB*, whereas mutations in the drug target DNA gyrase, in the genes *gyrA* and *gyrB*, were much less costly, and in the majority of cases, cost-free. This result was not surprising, as over-expression of an effux pump is more energetically costly than the mis-folding of the gyrase protein that results from the *gyrA* and *gyrB* mutations (Barnard & Maxwell 2001). Previous studies have shown that alterations to the drug target are selected first, and then additional mutations controlling drug accumulation further augment resistance (Jacoby 2005). Our data also fits this pattern, however, we would have to do further work to reconstruct the dynamics of substitution in these isolates through time in order to fully address this hypothesis.

4.4.2 Lack of significant epistatic interactions

Surprisingly, we have shown that epistatic interactions do not play a major role in determining the costs and benefits of acquiring multiple resistance mutations that evolved during the course of selection to ciprofloxacin in populations of *P. aeruginosa*. Specifically, we have shown that when combining a mutation in an efflux pump regulator and a mutation in the subunits of DNA gyrase, there is a trend for antagonistic epistatic interactions (Figure 4.4), however, in general these interactions are not statistically significant. We were surprised by this result because antagonistic epistasis between resistance mutations is routinely found between antibiotic resistance mutations to the same drug (Reynolds 2000; Rozen et al. 2007; Hall et al. 2011; Schenk et al. 2013) and pairs of resistance mutations that confer resistance to different drugs (Trinidade et al. 2009; Ward et al. 2009; Maclean et al. 2010; Borrell et al. 2013).

There are several differences between our study and the other studies examining epistatic interactions that might explain why we found very few instances of epistatic interactions. Most importantly, our study specifically focused on pairs of mutations that arose in a selection experiment, whereas the majority of other studies focused on mutations that arose either via a fluctuation assay or were introduced via molecular manipulations. Thus other studies assayed the potential for epistatic interactions between any resistance mutations, whereas our study measures realized epistatic interactions that are the result of selection to ciprofloxacin. To more fully explore this hypothesis, future work will examine the epistatic interactions in the full suite of double resistant mutants

(i.e. all 4 *nfxB* alleles in combination with all 4 *gyrA* alleles, and all 4 *gyrB* alleles; see "NA" boxes in Figure 4.5). These comparisons will allow us to contrast the difference in epistatic interactions between potential versus realized double resistant mutants.

Previously, pairs of ciprofloxacin resistance mutations have been shown to exhibit significant antagonistic epistasis, whereby costly single mutations in combination became less costly (Rozen et al. 2007). But all resistance mutations examined were within the drug targets *parC*, *parE* and *gyrA*, and thus the discrepancy between the results of this previous study, and our study, where we find very minimal epistatic interactions, may be because our study looked primarily at pairs of mutations comprising two different resistance mechanisms. Therefore epistatic interactions may be more prevalent between the same resistance mechanisms, and much less prevalent between different types of resistance mechanisms. However, in our study we did not find significant epistatic interactions between our only intra-mechanistic comparison (*gyrA* D87Y *gyrB* S466T).

The only case of significant antagonistic epistasis in our study was between an *nfxB* mutation and a *gyrB* mutation. This double mutant was comprised of the two single mutations that had the greatest mutational fitness effects. For many of the other double mutants assayed, the single mutations in *gyrA* and *gyrB* had fitness values close to one, and thus it is possible that these small fitness values impacted our power to detect epistasis.

Finally, both the strain (Borrell & Gagneux 2011) and assay environment (Hall et al. 2011) are factors that contribute to the epistatic interactions detected. As we only measured epistasis in a single genetic background and assay environment, future work

should investigate whether these epistatic interactions change in other strains of *P. aeruginosa* and across a range of environments.

4.4.3 Conclusion

Ciprofloxacin is one of the most common antibiotics used to treat *P. aeruginosa* infections in the lungs of cystic fibrosis (CF) patients. Clinical isolates from CF infections carry some of the same mutations that arose in our selection experiment; *gyrA* T83I, D87Y and D87N (Piddock 1999; Bruchman et al. 2012); *gyrB* mutations at codon 466 and 468 (Bruchmann et al. 2012), and mutations that knock out the function of *nfxB*, which in our experiment were the mutations fs161 and E75* (Wong & Kassen 2011). Therefore there is a direct applied aspect to our work. At the outset of this experiment we hypothesized that antagonistic epistasis would result in compensation when resistance mutations occurred together, supporting the view that epistasis between resistance mutations plays a large role in compensatory adaptation in pathogen populations. Our results would suggest that compensation of the cost of resistance might be a more complex process than previously thought, and involve genes external to those that confer resistance.

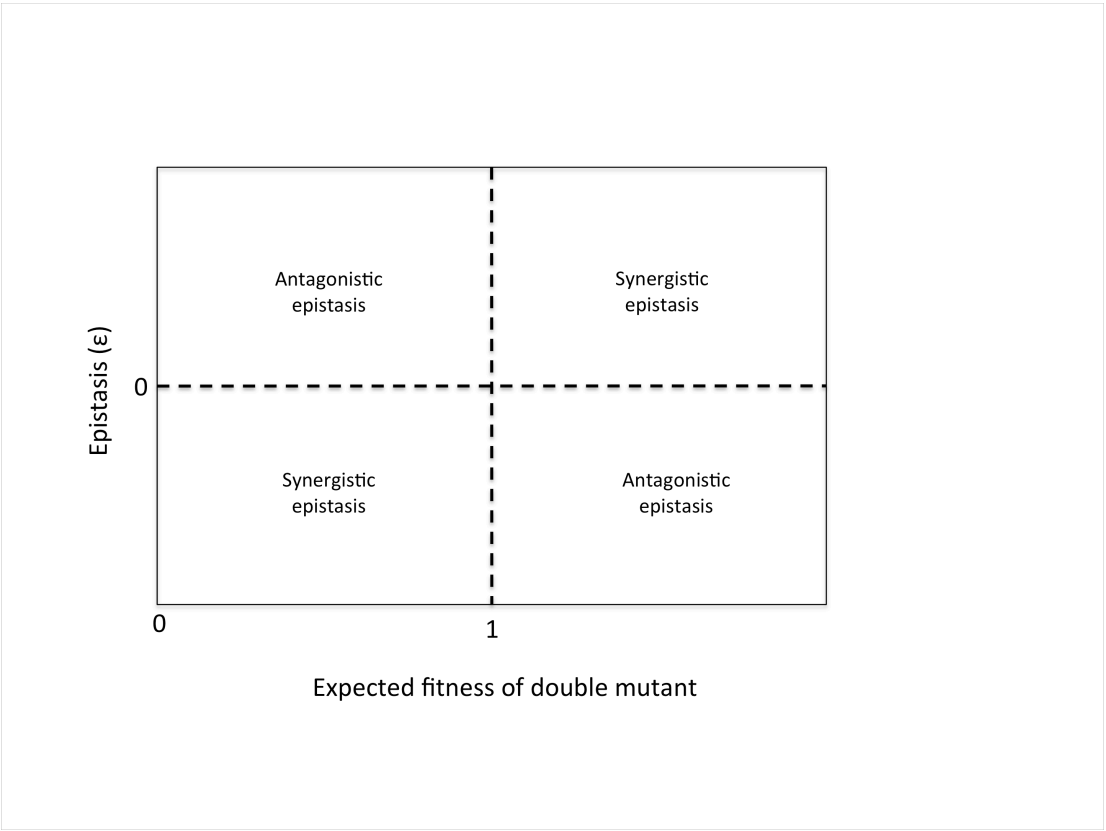

Figure 4.1. Quantifying antagonistic and synergistic epistasis between pairs of deleterious and beneficial mutations. See Table 4.1 for definitions. Adapted from Hall et al. 2011.

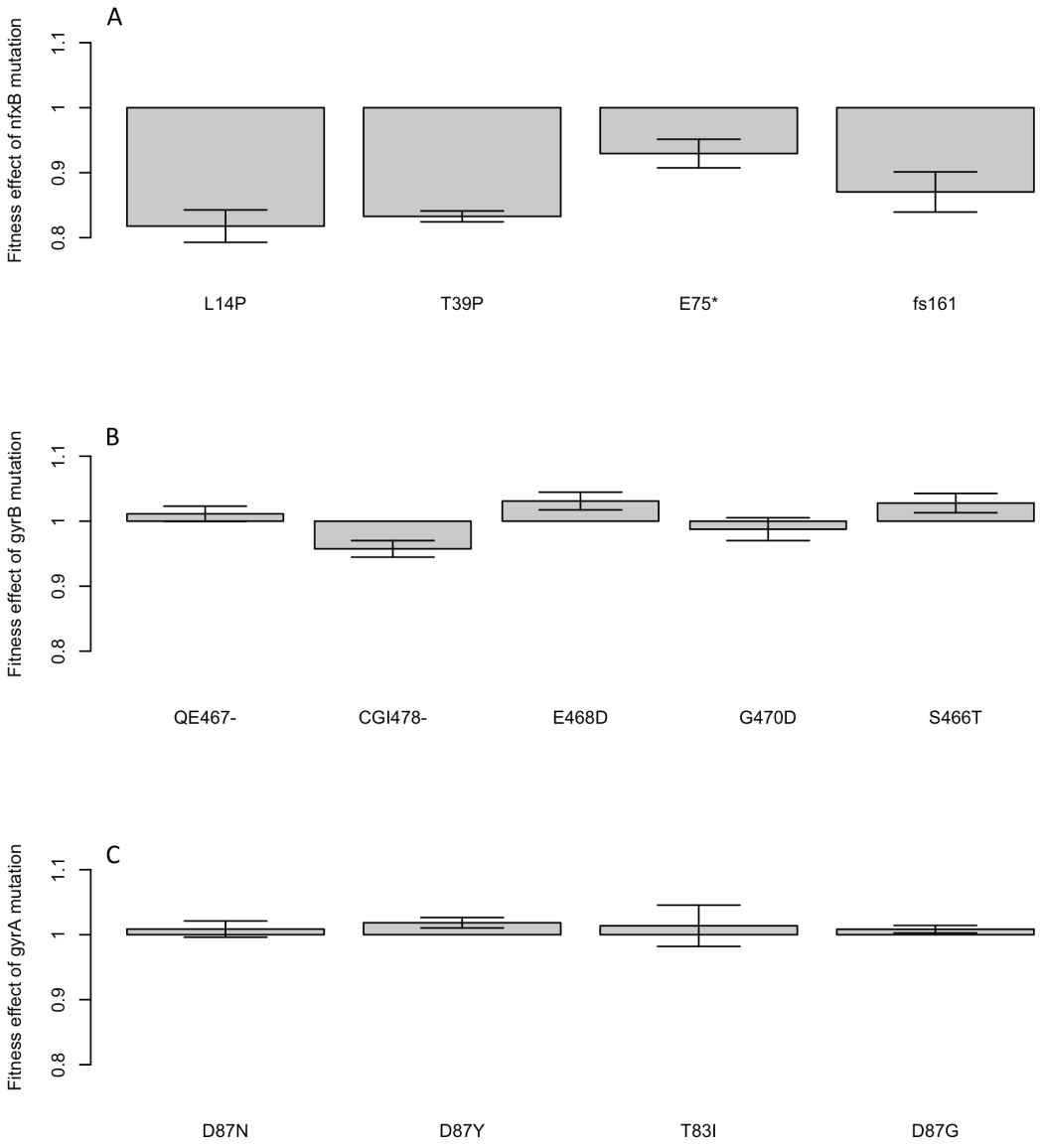

Figure 4.2. Mean fitness of each construct in (A) *nfxB*, (B) *gyrB* and (C) *gyrA* genes. Fitness of less than 1 represents a cost in the absence of the antibiotic. Error bars are +/- SE.

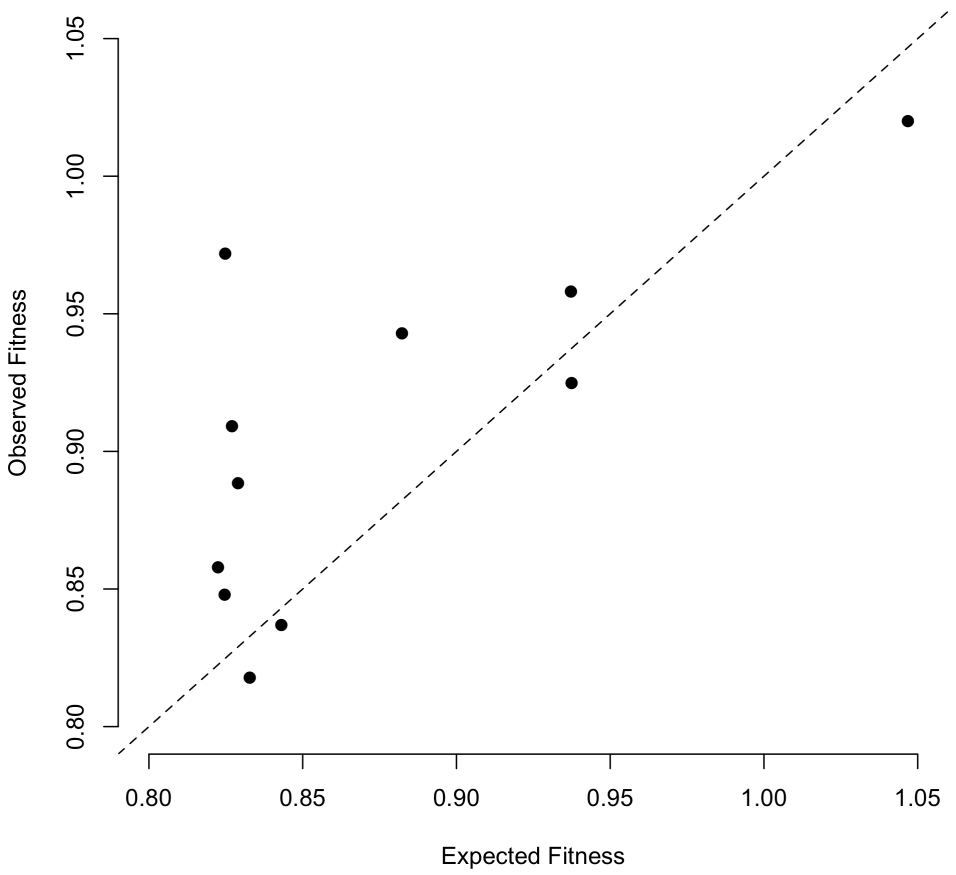

Figure 4.3. Observed vs. expected fitness of all double resistant mutants. The dashed line shows a 1:1 line.

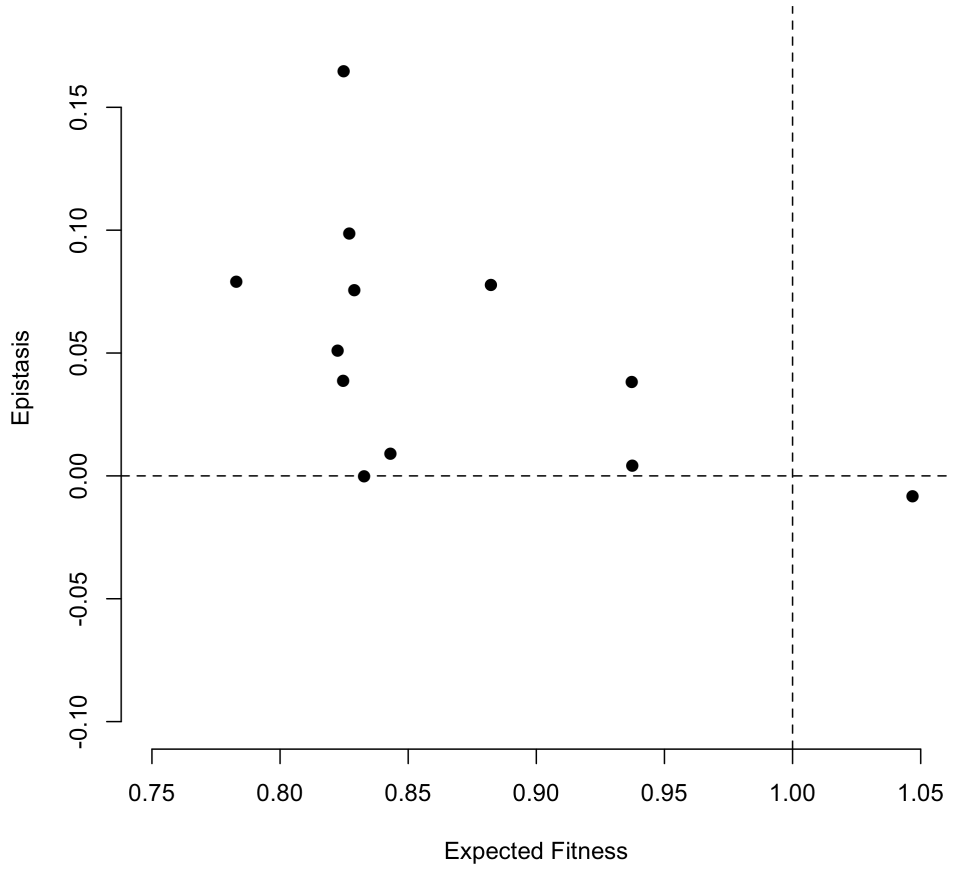

Figure 4.4. Epistasis between ciprofloxacin-resistance mutations, plotted against the expected fitness of double mutants based on the multiplicative fitness of each single resistance mutation. Each genotype (n=12) has two mutations (see Table C.1 for full list of combinations).

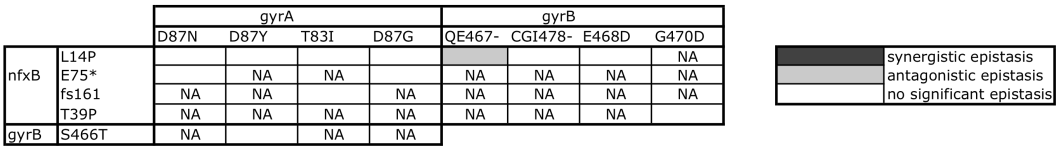

Figure 4.5. Epistatic interactions between double resistant mutants assayed, showing a lack of significant epistatic interactions. NA indicates we did not construct the double mutant for the two single mutations as it did not arise in the selection experiment.

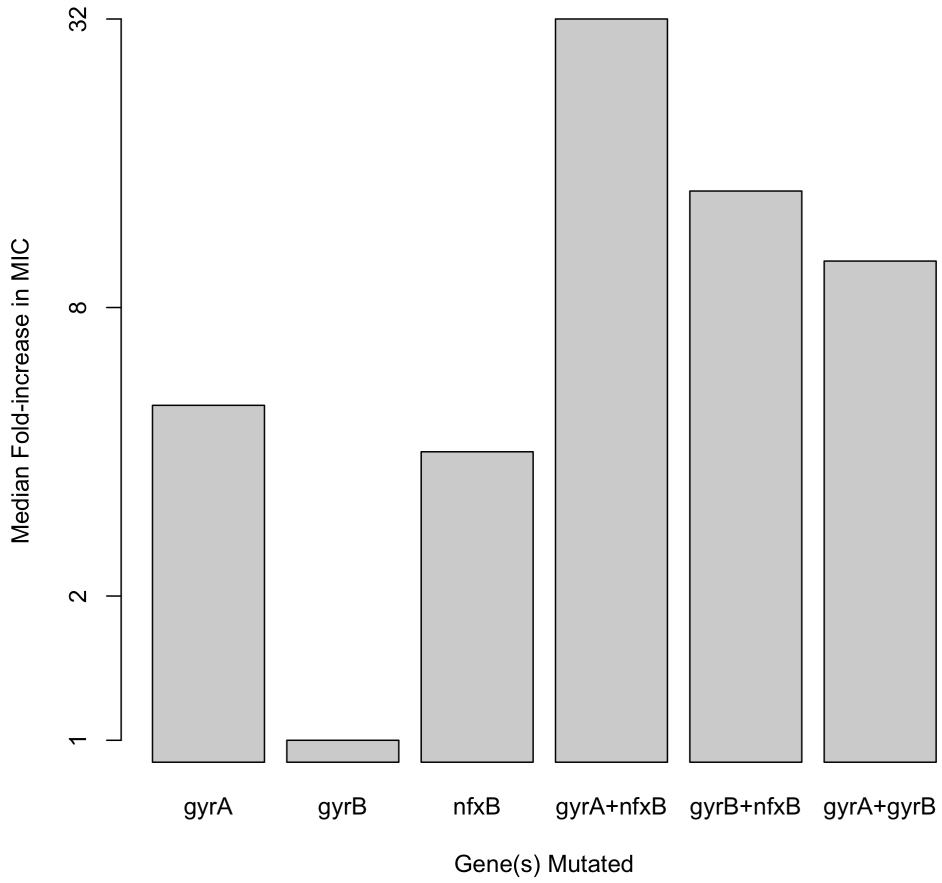

Figure 4.6. Median fold-increase in MIC for each single resistance gene, or double combination of genes.

Chapter 5

Comparative experimental evolution of antibiotic resistance in clinical strains of *Pseudomonas aeruginosa*

This chapter is reproduced from:

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Collaborator contributions:

J. Dettman performed all analyses on ancestral strains. The ancestral genomic data was previously published in Dettman et al. 2013; phenotypic measures were not previously published.

Abstract

The parallel evolution of antibiotic resistance, that is, mutations conferring resistance arising in multiple independent lineages within the same gene or at the same nucleotide, has been demonstrated in both laboratory studies and clinical settings. If resistance is conferred by mutations in only a small suite of genes, strategies to combat

the evolution of resistance can focus on a small number of known targets, whereas if resistance mutations vary across strains and are wide-ranging across the bacterial genome, it will be much more difficult to combat resistance evolution at the genomic level. Here, we examine the influence of genetic background on the evolution of resistance at the phenotypic and genomic levels by combining experimental evolution and comparative genome analysis. We selected for resistance to the antibiotic ciprofloxacin in four strains of *Pseudomonas aeruginosa* over approximately 200 generations of growth. All evolved isolates adapted and became resistant to ciprofloxacin; however, many isolates did not exhibit any mutations in the known resistance conferring genes. Indeed, strains isolated from chronic lung infections rarely evolved resistance through mutations in these known genes, indicating that we are missing a broad number of resistance mutations in clinical settings when we only screen a limited set of genes for mutations. We also show that the lack of parallel evolution between strains may be explained by differential mutation rates between strains at these known resistance sites.

5.1 Introduction

Parallel evolution is the independent evolution of the same phenotype or genotype in response to a shared selection pressure. There are examples of parallel evolution in the laboratory and in nature, particularly when studying the evolution of antibiotic resistance (e.g. Lieberman et al. 2011; Wong and Kassen 2011; Toprak et al. 2012; Wong et al. 2012; Dettman et al. 2013; Meinike Jorgensen et al. 2013; Vogwill et al. 2014; Melnyk et al. in prep) but also for other traits (Ostrowski et al. 2008; Rokas and Caroll 2008; Bailey

et al. 2015). Interestingly, many of these examples of parallel molecular evolution occur across genetic backgrounds, suggesting genetic background may be unimportant in determining the outcome of adaptation at the molecular level.

The lack of estimates of the influence of genetic background on parallel evolution can be at least partially explained by the different methods that have been used to study parallelism; within versus between genetic backgrounds (reviewed in Wood et al. 2005). Parallel evolution within a particular genetic background has often been experimentally tested using laboratory or greenhouse experimental evolution (reviewed in Wood et al. 2005; Wong et al. 2012; Meinike Jorgensen et al. 2013; Bailey et al 2015). In contrast, parallel evolution between different genetic backgrounds has been explored using phylogenetic or other comparative methods (reviewed in Wood et al. 2005; Lieberman et al 2011; Dettman et al. 2013; Caballero et al. 2015). There have been few attempts to incorporate comparative genomics and experimental evolution (but see Wichman et al. 2000; Holder & Bull 2001; Bollback & Huelsenbeck 2009; Vogwill et al. 2014).

By integrating experimental evolution and comparative genomics, it is possible to directly test the role of genetic background in determining the trajectory of adaptation. If differences in genetic backgrounds play an inconsequential role in adaptation, we would expect the same selection pressure to result in very similar outcomes across backgrounds. In this case, the extent of parallel evolution within-backgrounds should be equal to the probability of parallel evolution between-backgrounds. However, if genetic background is a key determinant of evolutionary potential, we expect adaptation to be highly contingent on genetic background. In this case the probability of within-background

parallelism should be much greater than the probability of between-background parallelism.

Box. 5.1. Can we use models of adaptation to predict the probability of parallel evolution?

Models of adaptation can be used to make predictions about the probability of observing parallel evolution at the level of genes or nucleotides during adaptation to a single, unchanging phenotypic optimum. Adaptation proceeds as a series of steps or moves in either phenotypic (Chevin et al 2010; Martin & Lenormand 2015) or genetic (Orr 2005) space caused by mutation that bring the population closer to the optimum, a process known as an adaptive walk. The crucial feature governing the probability of parallel evolution in these models is the spectrum of mutations available to selection; any factor that restricts the supply of beneficial mutations will increase the probability of parallel adaptation.

The effect of mutation supply on parallel adaptation is easy to appreciate in the simplest case of independently evolving populations founded from the same ancestral genotype adapting to a common environment. The supply of beneficial mutations will be the same initially for all populations, by definition, and, in the simplest scenario, the availability of beneficial mutations will decrease as adaptation proceeds and each population approaches the phenotypic optimum. The probability of parallel evolution is therefore expected to increase as a population adapts because there are fewer ways in which fitness can increase.

Now imagine that the starting strains are all similarly mal-adapted, in terms of

their distance to the optimum, but genetically distinct. The spectrum of mutations available to a particular strain will now depend on a range of factors in addition to distance to the optimum that can influence the rate and fitness effect of mutations. These factors include, for example, whether or not a given gene is present in the genome, its genomic location (Mira and Ochman, 2002), epistatic interactions among genes that make some kinds of mutation more or less likely (Weinreich et al. 2006; Maclean et al. 2010; Kvitek and Sherlock 2011), or the pleitropic effects of a mutation once they occur (Bataillon et al. 2011; Jasmin and Zeyl, 2013). As genotypes diverge ecologically and genetically, then, we expect their mutational spectra also to diverge. The probability of parallel evolution should therefore decline with increasing ecological and genetic disparity among genotypes.

Here we use comparative experimental evolution to test whether genetic differences between strains of a single species of bacterium influence the repeatability of adaptation to a shared selective pressure. Specifically, we selected for resistance to the widely use fluoroquinolone antibiotic ciprofloxacin, using ecologically and genetically distinct strains of the opportunistic pathogen *Pseudomonas aeruginosa*. We chose four genetically divergent strains based on a previously published whole-genome phylogeny (Dettman et al. 2013) and because they had comparable levels of resistance to our main selective agent, ciprofloxacin (Table 5.1). These strains are also expected to differ in ecology, with two strains, Pa01 and Pa14, being derived from acute wound infections and commonly used as representative pathogenic strains of *P. aeruginosa* by labs around the world, including our own (e.g. Ward et al. 2009; Maclean et al. 2010; Hall et al. 2011;

Wong et al. 2012; Bruchman et al. 2013; Gifford & Maclean 2013; Meinike Jorgensen et al. 2013; Gifford et al. 2015). The other two strains are clinical isolates derived from chronically infected cystic fibrosis (CF) patients in Ontario, Canada and are part of a group of understudied "epidemic" strains (Dettman et al. 2013; Aaron et al. 2010). *P. aeruginosa* isolates that chronically-infect CF patients tend to show reduced motility and virulence and higher levels of tolerance to oxidative stress and antibiotics compared to acute and environmental isolates (Smith et al. 2006; Wong and Kassen 2011; Marvig et al. 2013; Dettman et al. 2013; Marvig et al. 2014), reinforcing the view that the ecology of acute and chronic infections are substantially different.

Table 5.1. Ciprofloxacin resistance profile of *P. aeruginosa* strains used in this experiment. LES – Liverpool epidemic strain; ESB – Epidemic Strain B.

	Ciprofloxacin MIC (ug/mL)
Pa14	0.5
Pa01	2
JD313 (LES)	4
JD322 (ESB)	1

The fluoroquinolone antibiotic ciprofloxacin is often used in treatment of lung infections in CF patients (Høiby et al. 2010, Sordé et al. 2011), and high levels of resistance are rapidly detected post-treatment in *P. aeruginosa*. Fluoroquinolone resistance typically occurs via one of two main mechanisms (Ruiz 2003; Jacoby 2005). First, mutations in negative regulators (*nfxB*, *mexR*) of multidrug efflux pumps (MexCD-

OprJ, MexAB-OprM) result in increased expression of these pumps, resulting in decreased intracellular concentrations of fluoroquinolones (Poole 2005). Loss of function mutations across these regulator genes has been shown to confer resistance (Wong et al. 2012). The second main mechanism of resistance is via mutations that prevent the fluoroquinolone from binding to its molecular targets, DNA gyrase and topoisomerases. Mutations in this case are much more targeted, typically in regions of the quinolone-resistance determining region (QRDR) in gyrase A, specifically at codons 83 and 87 (reviewed in Wong and Kassen 2011; see also Piddock 1999; Bruchman et al. 2013; Wong et al. 2012; Melnyk et al. in prep), and also at various sites within gyrase B, and topoisomerases *parC* and *parE* (Yoshida et al. 1990a,b; Barnard and Maxwell 2001). In addition to these known resistance-conferring genes, new, putative resistance-conferring genes are being continually discovered (Wong et al. 2012). Therefore parallel evolution is possible but is not universal.

Here we use a comparative experimental evolution design that involves selecting replicate populations starting from genetically unique starting strains of *P. aeruginosa*. After 200 generations of selection for ciprofloxacin resistance, we examined isolates from evolved populations and quantified the phenotypic outcome of selection by measuring the minimum inhibitory concentration (MIC) of ciprofloxacin, and the growth rate in both the presence and absence of ciprofloxacin. The genomic outcome of selection was measured by identifying all mutational changes that were present in each evolved isolate at the end of the experiment.

5.2 Results and Discussion

This experiment was designed to evaluate the repeatability of the genetic causes of ciprofloxacin resistance. Additionally, we wanted to test whether the probability of parallel genomic evolution declines with increasing ecological and genetic divergence among genotypes. Populations of *P. aeruginosa* were evolved in lysogeny broth (LB) supplemented with ciprofloxacin for approximately 200 generations of selection. The experiment comprised 4 genetically distinct strains of *P. aeruginosa*, with 8 replicate evolving populations per strain, for a total of 24 experimental populations.

5.2.1. Phenotypic adaptation

Selection in ciprofloxacin led to the evolution of resistance in all evolved populations, regardless of founding strain (t_{23}=2.91, P<0.01). Variation in final resistance was significantly explained by founding strain (Figure 5.1; F=4.06, P<0.001). Evolved Pa14 isolates had significantly greater fold-increases in resistance than isolates from other founding strains (Table D.1).

To investigate whether resistance acquisition was associated with a fitness cost in the absence of antibiotic, we compared growth rates of the evolved isolates in the absence of ciprofloxacin to the growth rates of their respective founding strains. Growth rates were measured as the mean of three replicates, and standardized against the mean growth rate of Pa14 ancestor control wells that were included in each plate assay. There was a significant difference in ancestral growth rates (F=99.9, P<0.001), with all strains being

significantly different from one another (Pa01>322>Pa14>313; Table D.2). Evolved isolates grew significantly more slowly than their respective ancestral strains (Figure 5.2 (A); Table D.3), with the exception of Pa14 that had no significant change in mean growth rate (t_7=1.38, P=0.184) in the absence of ciprofloxacin. In general a fitness cost was associated with resistance acquisition, such that strains had slower growth in the absence of antibiotic even after 200 generations of selection to laboratory conditions.

To investigate the direct effects of selection, we assayed the growth rate of evolved isolates in the presence of ciprofloxacin. All evolved isolates were more fit in this environment, as ancestral strains grew at approximately 20% of full growth in LB (see Materials and Methods). Isolates from each founding strain did not differ significantly from one another in growth rate in the presence of ciprofloxacin after 200 generations of selection (Figure 5.2 (B); F=1.87, P=0.168), indicating that they all adapted to the selection environment to a similar extent.

5.2.2. Genetic adaptation

To gain insight into the genetic causes of ciprofloxacin resistance, we sequenced the genomes of a single isolate from each of the 24 evolved populations. We identified two adaptive genetic strategies, either to become a mutator or not. This was not unexpected, as previous work has shown becoming a mutator can be adaptive in the presence of antibiotic stress *in vivo* (Meinike Jorgensen et al. 2013), and mutator strains are often observed within the lungs of CF patients (Oliver et al. 2000; Oliver et al. 2004; Smith et al. 2006; Marvig et al. 2013; Marvig et al. 2014). Mutator strains were excluded

from this manuscript, as it is not possible to compare estimates of parallel evolution between mutator and non-mutator strains. One evolved isolate from Pa14 and three evolved isolates from JD313 had mutations in *mutS*, resulting in an increased mutation rate because of their deficient mismatch repair system (Oliver et al. 2002). We also included a 5th founding strain in our experiment, JD334, however, all evolved isolates from this strain were mutators and so we excluded this founding strain from the analyses. We do not have sequence data for one isolate descended from JD313 due to low quality sequencing (Table D.4).

Mutator strains aside, we found 0 to 9 (mean=3.80) mutations per evolved isolate, with a mean sequencing coverage of ~53-fold. The majority of mutations (n=46) were SNPs, but we also found a fair number (n=36) of deletions and 13 insertions (Table D.5 lists all mutations relative to PA14 reference genome number NC_008463.1, and the mutation's predicted functional consequences). Together these mutations represent 72 unique changes affecting a total of 52 genes. No large insertion or deletion events were found.

Figure 5.3 shows the mean number of mutations per evolved isolate for each founding strain. There was a significant effect of strain on the number of mutations that arose (F=8.91, P<0.001). Interestingly, isolates from strains frequently studied in the lab that are from acute infections, Pa14 and Pa01, had significantly more mutations than isolates founded from clinical strains JD313 and JD322 (Table D.6). The number of mutations observed at the end of the selection experiment did not significantly explain variation in MIC (F=1.14, P=0.297), or variation in growth rate in the presence (F=2.64, P=0.118) or in the absence (F=2.31, P=0.142) of ciprofloxacin.

5.2.3. Unique genetic basis of adaptation to ciprofloxacin

Resistance to fluoroquinolones such as ciprofloxacin typically occurs by two main mechanisms: increased activity of multidrug efflux pumps via mutation in their regulators (*nfxB* and *mexC;* Poole 2005), and alterations of the drug target by mutations at key sites in the genes encoding DNA gyrase (*gyrA* or *gyrB*) and DNA topoisomerase (*parC* and *parE*; Barnard & Maxwell 2001; Yoshida et al. 1990). Therefore *a priori* we expected a strong signal of parallel evolution, driven by multiple mutations occurring in known antibiotic resistance conferring genes, specifically in the genes *gyrA*, *gyrB*, and *nfxB* that have been frequently mutated in previous studies (reviewed in Wong and Kassen 2011; see also Piddock 1999; Bruchman et al. 2013; Wong et al. 2012; Melnyk et al. in prep). In this study, of a total of 95 identified mutations, we found 22 mutations that were within known ciprofloxacin-resistance conferring genes; 17 mutations in *gyrA* and *B*, 5 mutations in *parC* and *E* and only 6 mutations in the efflux pump regulators *nfxB* and *mexC*. Interestingly, 9 evolved isolates that were resistant to ciprofloxacin had no mutations in any gene previously associated with resistance to ciprofloxacin. This would indicate that mutations in additional genes within the *P. aeruginosa* genome confer ciprofloxacin resistance. This result was surprising as many of the most striking examples of parallel molecular evolution across genetic backgrounds come from the evolution of resistance to toxins such as insecticides (Constant et al. 2004), herbicides (Bernasconi et al 1995) and antibiotics (Jatsenko et al. 2010), and therefore we expected

strong antibiotic selection pressure to result in increased parallel evolution among all strains based on common shared mutations in known resistance genes.

Interestingly, although we did see mutations in previously described canonical resistance genes (Figure 5.4; top box containing *gyrA*, *gyrB*, *parC*, *parE*, *nfxB*, and *mexC*), in isolates descended from Pa14 and Pa01, isolates descended from clinical strains JD313 and JD322 had very few mutations in these genes, indicating resistance to ciprofloxacin was from mutations within novel resistance genes. Therefore, unexpectedly, the genetic basis of ciprofloxacin resistance seemed to differ between founding strains and there is evidence for past ecology playing a role in determining the mutations that arise and confer ciprofloxacin-resistance.

5.2.4 Why the lack of mutations in known resistance genes?

I) Is the MexCD-OprJ pump already constitutively on in some ancestral strains?

A possible explanation for the lack of mutations in *nfxB*, the negative regulator of the MexCD-OprJ efflux pump, in isolates founded from Pa01, JD313 and JD322 is that the MexCD-OprJ efflux pump is already constitutively turned on in these strains, and thus mutations in *nfxB* would have no further effect on growth in the presence of the antibiotic. To test this hypothesis we used the efflux pump inhibitor phenylalanine-arginine beta-naphthylamide (PAbN) and tested for the difference in MIC of each strain in the presence and absence of PAbN (Bataillon et al. 2011) with three different drugs belonging to three different classes of drug; ciprofloxacin (fluoroquinolone), ceftazidime (cephalosporin) and gentamycin (beta-lactamase). If any efflux pump were already

constitutively expressed in these strains, we would expect the ratio of the MIC in the absence: MIC in the presence of PAbN to be less than one. We found that for all drugs assayed, the ratio of the MIC in the absence and presence of PAbN was never less than one (Figure D.1), indicating that none of these strains have efflux pumps that are constitutively on. Therefore mutations in efflux pump regulators are still likely to have an effect in the strains used in this study, and ancestral differential regulation of efflux pumps does not explain differences in the frequency of mutations occurring in their regulators between strains in this experiment.

II) Genome alignment

Another possible explanation for the mutational differences between the commonly used laboratory strains and the CF strains may be an alignment issue. While we aligned the acute strains to fully closed genomes (as they are widely used laboratory strains for which this data is readily available), we had only scaffold alignments for the CF collection of strains. While it is impossible to enumerate the percentage of each genome we are missing, we did check to ensure that we have sequence for all canonical resistance genes. For all genes relating to resistance in Figure 4, we checked that (i) they were present in the ancestral scaffold alignment, and (ii) that we have good coverage (>30 sequences) across these genes. All CF isolates passed these two sets of criteria, and thus the lack of mutations at in these known genes was not because of missing regions of the genome. We also went one step further and checked for mutational changes at codon sites 83 and 87 in *gyrA* that frequently arise and confer resistance to fluoroquinolones. No

isolates from JD313 and JD322 showed any of the expected changes associated with fluoroquinolone resistance; T83I, D87G, D87N, D87Y **(**reviewed in Wong and Kassen 2011; see also Piddock 1999; Bruchman et al. 2013; Wong et al. 2012; Melnyk et al. in prep**)**. Therefore genomic variation, at least at known resistance conferring genes, is not due to alignment issues or low coverage.

5.2.5 Candidate novel ciprofloxacin-resistance conferring genes

For the 9 isolates that did not have a mutation in a canonical resistance gene (Figure 5.4) we surveyed the function of all genes that did have a mutation. Several candidate genes present themselves. Three isolates (JD313-7; JD322-1; JD322-5) have mutations in PA14_32420, encoding a putative oxidoreductase, which was previously suggested to confer resistance to ciprofloxacin in Pa14 (Wong et al. 2012). Two isolates, JD322-2 and JD322-3, have mutations in *topA*, a DNA type I and II topoisomerase (Heddle et al. 2001), which may have similar binding affinity to the type II topoisomerase *gyrA* and *parC*. Previous studies that examine adaptation to conditions within the lungs of CF patients may yield other candidate adaptive genes. One gene, *ygdP*, overlapped with those found in a screen of transposable-element insertions for novel ciprofloxacin determinants (Breidenstein et al. 2008). Two genes external to canonical resistance genes, *wbpM* and *morA,* overlapped with those identified in CF clinical strains to be under selection by Marvig and colleagues (2014). No mutated genes in our study were the same as the pathoadaptive genes identified by Caballero and colleagues (2015). Future work

will use allelic replacements of candidate mutations in these lines to identify the mutation(s) and gene(s) conferring ciprofloxacin resistance.

5.2.6. Quantifying genomic parallel evolution

The most striking result of our study is the lack of parallel evolution to a strong, common antibiotic selective pressure. To directly quantify the extent of genomic parallel evolution, we calculated the mean Jaccard index, *J*, for within and between founding strain comparisons (Wong et al. 2012; Bailey et al. 2015). *J* ranges from 0 to 1, with 0 indicating no parallel evolution and 1 indicating identity (see Materials and Methods). We found that parallel evolution, while low, was generally more common within isolates that shared the same founding strain than between isolates from different founding strains, implying that genetic background has a small but detectable impact on adaptation in this system. Vogwill et al. (2014) also found higher parallelism within versus between strains when using a comparative experimental evolution approach to studying rifampicin resistance evolution in *P. aeruginosa*. However, in our study the magnitude of this effect was much more varied, as parallelism within genetic backgrounds varied from no parallelism between strains founded from in JD313 to 31% of mutations in strains founded from Pa14. Overall, even between isolates from a common ancestor, the occurrence of the mutations within the same gene in multiple independent evolving lines is very low, indicating that most isolates are on unique evolutionary trajectories. This result was unexpected, as strong selection to the antibiotic ciprofloxacin has previously been shown to result in resistance acquisition through mutations in several known genes.

5.2.7 Factors influencing the probability of genomic parallel evolution:

I) Distance from the Optimum

Several factors may influence the probability of parallel evolution (Box 5.1), either within or between starting strains. One possible factor, identified in recent theoretical work by Chevin et al (2010) and Orr (2005) suggests that there should be a negative relationship between the degree of adaptation and the probability of parallel evolution. This draws from the simple rationale that a mal-adapted population located some distance form a fitness optimum has many more possible beneficial mutations available than one located very near to the optimum itself. To examine this idea within the context of our experiment we asked whether there was a correlation between growth rate in LB + ciprofloxacin, a proxy for distance from the optimum, where higher growth rates corresponding to a population being closer to the optimum, and the probability of parallel evolution. There was no correlation between distance from the optimum and the probability of parallel evolution, as measured by the mean *J* within a strain (χ=-0.567, *P*=0.433; Figure 5.5). Thus the low degree of parallel evolution cannot be explained by differences in distance to the optimum.

II) Genetic and Ecological Similarity

The probability of parallel evolution between strains may also be partially explained by how similar strains are, either genetically or ecologically (i.e. niche

overlap). One might expect the probability of between strains parallel to be correlated with genetic similarity between strains. However, there is no correlation between genetic distance between starting strains and the probability of parallel evolution between strains in our experiment (χ=0.351, *P*=0.494; filled circles in Figure 5.6A) indicating that parallel evolution is not more likely between populations that are more closely genetically related.

At the phenotypic level we expect strains to differ in ecology; JD313 and JD322 were isolated from chronic infections within the lungs of CF patients, whereas Pa14 and Pa01 were recovered from acute wound infections. Previous work has shown that niche adaptation is a major evolutionary force influencing the composition of *P. aeruginosa* genomes (Mathee et al. 2008). Specifically, the CF airway is characterized by multiple disparate sources of oxidative stress, for example oxidative bursts by host macrophages lead to increased nitric oxide and superoxide levels (MacMicking et al. 1997), and the lungs of CF patients also have reduced levels of the major pulmonary antioxidant glutathione (Roum et al. 1993). Thus CF strains of *P. aeruginosa* have adapted to living in an environment that has increased oxidative stress. Niche adaptation, to conditions of oxidative stress or other environmental factors, is likely to result in phenotypic differences between strains. Therefore to determine how different these strains are phenotypically, we measured the Euclidean distance between strains in our experiment using 10 phenotypic trait measurements (Materials and Methods). There was no correlation between Euclidean distance between strains and the probability of parallel evolution between strains (χ=0.103, *P*=0.845; filled circles in Figure 5.6B) indicating that parallel evolution is not more likely between populations that are more ecologically

similar. Interestingly, genetic distance and phenotypic distance were also not correlated, indicating that among these strains of *P. aeruginosa,* phylogeny does not predict ecology (χ=0.296, *P*=0.569; Figure 5.6C). Thus in our study, historical contingency, measured at both the genetic and phenotypic levels, does not explain the lack of genomic parallel evolution in our study.

III) Mutation rate heterogeneity

Finally, it is possible that the low probability of parallel evolution between strains in response to strong antibiotic selection pressures is because of variation in mutation rates across strains at different sites within the genome. Models show that under certain conditions mutation rate heterogeneity has a role in governing the probability of parallel evolution (Chevin et al. 2010), and it is known that in *P. aeruginosa* mutation rate varies along the replichore (Dettman et al. 2016; Mira and Ochman 2002), however few studies have investigated differential mutation rates between strains and at different sites of interest. That being said there is some evidence for differential mutation rates having a role in ciprofloxacin adaptation. A study by Wong & Seguin (2015) identified differences in substitution rates between different *Escherichia coli* strains adapting to ciprofloxacin.

To test for differential mutation rates we used a fluctuation test to measure mutation rate of each strain, and to approximate the proportion of mutations conferring ciprofloxacin-resistance attributable to known resistance-conferring genes. The lack of parallel evolution at characterized antibiotic resistance genes may be the result of differential mutation rates either (a) between strains overall, and, perhaps most

importantly (b) at the canonical resistance-conferring genes (*gyrA*, *gyrB*, *nfxB*, *parC* and *parE*) between strains. Briefly, we tested 96 replicate populations from each founding strain, and we sequenced between 28-47 ciprofloxacin-resistant mutants at the five antibiotic resistance conferring genes previously identified, to determine the proportion of mutations within each gene that are contributing to resistance. We used the Ma-Sandri-Sarkar (MSS) Maximum Likelihood Method (Sarkar et al. 1992; Ma et al. 1992) in the FALCOR program (Hall et al. 2009) to determine overall strain mutation rate. We also tested the robustness of our results by approximating mutation rate using the Lea-Coulson method of the median and a frequency based calculation, and all measures gave approximately the same mutation rates for all strains (data not shown). JD313 had a higher mutation rate approximately an order of magnitude greater than the other three strains used in this experiment (Table 5.2). This may explain the lack of parallel evolution within strains founded by JD313, as a higher mutation rate would result in a greater number of mutations being sampled throughout the course of our selection experiment, if mutation supply is limiting.

Table 5.2. Mutation rate (m/Nt) and associated 95% confidence intervals, calculated via the MSS maximum likelihood method.

Strain	Mutation rate	95% Confidence Interval
Pa14	0.0044	0.0034-0.0056
Pa01	0.0047	0.0036-0.0059
JD313	0.0463	0.0366-0.0569
JD322	0.0046	0.0035-0.0059

We also sequenced resistant mutants from the fluctuation test to identify differences in the number of mutations that arose within five known resistance-conferring genes, between strains. Surprisingly, there were very few mutations in the five genes sequenced (Table 5.3). Interestingly, Pa14, JD313 and JD322 only had mutations in *nfxB*, while Pa01 had a single mutation in *gyrA*. This result is noteworthy as it indicates that we may need to expand our view of the evolution of antibiotic resistance to include differential mutation rates at known target genes. Additionally, the sequencing results confirm that we are still unaware of several genes that confer ciprofloxacin-resistance in *P. aeruginosa*, as the majority of resistant strains in this assay did not have mutations within these known resistance-conferring genes.

Table 5.3. The proportion of ciprofloxacin-resistant mutants that had mutations in each seqeuenced gene, by strain.

	gyrA	gyrB	parC	parE	nfxB
Pa14	0	0	0	0	0.179
Pa01	0.022	0	0	0	0
JD313	0	0	0	0	0.045
JD322	0	0	0	0	0.098

5.2.8 Conclusions

This study was designed to evaluate the repeatability of the genetic causes of antibiotic resistance. We used experimental evolution of four strains of the opportunistic human pathogen *P. aeruginosa* adapting to a common selective environment containing the fluoroquinolone antibiotic ciprofloxacin. We found that all evolved isolates were resistant, but that resistance was not always conferred by mutations in known resistance-conferring genes. Strains from acute infections that are often used in lab experiments, Pa14 and Pa01, gain resistance to ciprofloxacin via mutations in a suite of known resistance genes, whereas chronic strains of *P. aeruginosa* did not have mutations in any known resistance-conferring genes.

Additionally, we examined the probability of parallel evolution at the genomic level. We know that the spectrum of mutations available to a particular strain will depend on a range of factors that influence the rate and fitness effect of mutations (Box 5.1). In our experiment we found that ecological and genetic divergence did not correlate with probability of parallel evolution, and unexpectedly neither did distance from the optimum. While this lack of correlation may partially be due to low sample size, it may also indicate that divergence between strains at some scales is less important than other more proximate factors such as mutation rate and epistatic interactions between genes under selection. We did find that mutation rates differed between strains at known resistance conferring genes, indicating that at this level of divergence between strains, mutation rate heterogeneity within the genome may play a key role in determining evolutionary trajectories, and thus the probability of parallel evolution. We also

hypothesize that epistatic interactions may occur between resistance mutations and other genes within the genome such that the fitness effects of known resistance mutations are markedly different between strains (Borrell and Gagneux 2011). Future work will use allelic replacement constructs to test the fitness effects of known ciprofloxacin-resistance mutations across genetic backgrounds within *P. aerugionsa*.

A key result of this experiment is that many mutations in more genes than expected can drive the evolution of ciprofloxacin resistance in *P. aeruginosa*. As genome sequencing continues to become faster and more inexpensive, evolution experiments using a broad number of strains for many clinically important pathogenic bacterial species will enable us to see whether this trend holds at a broader scale.

5.2.9 Broader Implications

It is common practice in many fields of genetics to use one or a few standard laboratory strains to make inferences about the genetic architecture of traits or phenotypes of interest that characterize the entire species or group. The underlying and often unspoken assumption here is that what is true for the strain being studied in the laboratory is also true for all or most other strains within the species. The justification for this assumption is that strains within a species, or species within a closely related group or taxon, share a common evolutionary history and so a common genome. How one particular strain deals with a common stressor should then be a good guide as to how other members of the group deal with the same stressor.

While this assumption may often be justified in cases where the genome remains essentially unchanged, it is not likely to hold when those stressors are sufficiently strong to generate evolutionary change. The stochastic and sometimes idiosyncratic nature of genetic variation, especially when generated by mutation, means that the evolutionary response of a lineage to stress cannot reliably be predicted by homology alone. This problem is especially acute in situations where rapid evolution is likely to occur, such as to antibiotic stress, as the course of future evolution may depend intimately and in unpredictable ways on the routes that were followed in the past.

The results of our study clearly suggest that making broad inferences about the genomic causes of antibiotic resistance based on the results of studies using a single bacterial strain may not be representative of the evolutionary changes that may occur within the bacterial species as a whole. Notably, our study indicates we may be vastly underestimating the genomic causes of antibiotic resistance by often only studying a single, representative pathogenic strain.

5.3 Materials and Methods

5.3.1. Experimental evolution

Five different strains of *P. aeruginosa* were used in this study: lab strains Pa14 and Pa01 and clinical isolates JD313, JD322 and JD 334 (Dettman et al 2013). The clinical strains were isolated from CF patients in Ontario (Aaron et al 2010; Dettman et al 2013). Single colonies of each of the five different strains were grown overnight in

Lysogeny broth (LB; bacto-tryptone 10g/L, NaCl 3 g/L, yeast extract 5 g/L) to start the experiment. Eight populations were founded from each progenitor by adding 20 uL of overnight culture to 1.5 mL of selection media. An aliquot of progenitor was frozen at -80^{o}C in glycerol. Populations were grown in an orbital shaker (150 rpm) at 37^{o}C for 24-hour cycles in 24-well plates (Corning, NY, USA). After 24 hours each population was serially propagated by transferring 20 uL of overnight culture to 1.5 mL of fresh selection media. Overnight cultures were frozen every eight transfers at -80^{o}C in glycerol (%vol), such that approximately 50 generations of adaptation occurred each freezing cycle.

Selection media was LB supplemented with the fluoroquinolone antibiotic ciprofloxacin. The concentration of ciprofloxacin was the concentration that reduced founding strain growth to 20% of full growth in LB. Ciprofloxacin concentration for each strain were as follows; Pa14 0.8 ug/mL; Pa01 1.2 ug/mL; JD312 0.3 ug/mL; JD313 1.2 ug/mL; JD322 0.8 ug/mL; JD334 2.4 ug/mL. Three populations founded from Pa14 and three populations from JD334 were contaminated at the end of the experiment and were excluded from analysis (Table 1).

5.3.2. Phenotypic analyses

From each evolved population a single isolate of the most common type was obtained. For each evolved isolate, the level of resistance was assayed as the minimum inhibitory concentration (MIC) of ciprofloxacin. Overnight cultures of each evolved isolate were grown in LB broth, of which 10 uL was diluted into 10 mL LB broth and 100 uL of this dilution was used to inoculate 96-well plates with varying concentrations of

ciprofloxacin. For each evolved strain we assayed growth at 0x, 1x, 2x, 4x, 8x, 16x, 32x, 64x, 128x, 256x, and 512x the ancestral MIC. Log-transformed MICs were used for all analyses.

Fitness of the evolved strains in the presence and the absence of the ciprofloxacin were measured as the maximum growth rate of each strain over a 24-hour period. For each assay, 20 uL of overnight culture was added to 180 uL of LB (with or without ciprofloxacin at the concentration used in the selection experiment) and the optical density was measured by spectrophotometry every 90 minutes. Growth rate during the exponential phase was then estimated using Gen5 software (Bio Tek Instruments Inc., Winooski, VT), and fitness was taken as the mean growth rate of 3 replicates. It has been previously shown that fitness of antibiotic resistance mutations estimated by growth rates in *P. aeurginosa* is strongly correlated with fitness estimated by pair-wise competition assays (Perron et al. 2010). Growth rate values were standardized by making them relative to the growth rate of 12 control wells of ancestral Pa14 grown in LB for each experimental plate. Log-transformed mean growth rates were used for all analyses (Houle et al. 2011).

5.3.3. Whole-genome sequencing and analysis

For whole-genome sequencing, each evolved isolate used in the above analysis was grown overnight in LB, and genomic DNA was extracted using the Qiagen DNEasy DNA purification kit. Genome Quebec Innovation Centre performed 250-bp paired-end Illumina sequencing using the Mi-Seq platform. Mean coverage across all 16 genotypes

was ~53-fold per genotype (mean=53.1; range 36.2-67.1). The sequence data were aligned to either the ancestral reference genomes (Pa14 reference genome number NC_008463.1, Pa01 reference genome number NC_002516), or in the case of the JD clinical isolates to draft genomes which were scaffold alignments made using ProgressiveMauve (Darling et al. 2010, version 2.3.1) from Dettman and colleagues (2013: JD313 AWYP00000000; JD322 AWYV00000000; JD334 AWZF00000000). Mutations were called and annotated using a custom computational pipeline (modified from Dettman et al. 2012), and then all mutations alignments were checked visually using SamTools (ver. 0.1.19). One isolate founded from JD313 was excluded based on poor quality of sequence data (Table 1).

5.3.4. Parallel evolution

We quantified similarity in genetic changes between pairs of populations using the Jaccard Index, *J* (Wong et al. 2012; Bailey et al. 2015). Given two sets G1 and G2 of mutation-bearing genes found in isolates 1 and 2, respectively,

$$J(G1,G2) = \frac{(G1 \cap G2)}{(G1 \cup G2)} \tag{5.1}$$

That is, J is the number of genes mutated in both populations, divided by the total number of genes mutated in isolate 1 or in isolate 2. J ranges from 0 to 1, with 1 indicating identical genotypes and 0 indicating no shared mutation-bearing genes. This value was calculated for all pairs of evolved isolates, both within and between founding strains. As

parallelism was low at the gene-level we did not compute nucleotide-level Jaccard indices.

5.3.5. Annotation of gene function

Annotation of gene function was done using the Pseudocap function as listed on the Pseudomonas genome database (Winsor et al. 2011). We supplemented the "antibiotic resistance and susceptibility " class with further genes that were known to be involved with fluoroquionlone resistance. These included *gyrA*, *gyrB*, *parC*, *parE*, and *nfxB* (Poole 2005; Barnard and Maxwell 2001; Yoshida et al. 1990). Additionally, cells with crosshatched notation for antibiotic resistance and susceptibility indicate genes that have been hypothesized to be involved with fluoroquionlone resistance (*morA*, *orfN*; Wong et al. 2012).

5.3.6. Ancestral analysis

As a measure of genetic divergence between strains we used Kimura-2-parameter genetic distances based on 5058069 bp core genome alignment of 32 strains of *P. aeruginosa* (Dettman et al. 2013). As a measure of phenotypic divergence between strains we used the Euclidean distance between strains based on 10 measured phenotypic traits, using the dist() function in R.

To characterize the phenotype of ancestral strains we measured 12 traits for each strain; we measured the minimum inhibitory concentration of the three antibiotics

ciprofloxacin, gentamycin and ceftazidime, as well as 2 salts, $CuSO_4$, and NaCl. We measured strains swimming and swarming ability, as well as their mucoidy, autolysis and iridescence. We also measured pyocyanin and pyoverdine production.

MIC assays were performed via the same method as above, but concentrations were recorded as ug/mL and not fold-increase. Ciprofloxacin MIC was not included as a phenotype measure because all strains in our experiment had similar MICs as this was the trait for which we had selected them for in our experiment. We assessed ancestral strain ability to swim and swarm. To assess swimming ability strains were diluted to 25% of full growth and 5 uL of dilute culture was stabbed into the middle of modified LB agar plates (50% NaCl and 0.3% agar). After 48 hours of incubation at 37^0C the turbid zone radius was measured and recorded. To assess swarming ability strains were diluted to 25% of full growth and 10 uL of dilute culture was dropped onto the surface of modified LB agar plates (50% NaCl, 0.5% agar and 0.5% glucose). Swarm radius was measured after 48 hours of growth at 30^0C. Mucoidy, autolysis and iridescence was measured by spotting full growth culture onto *Pseudomonas* isolation agar (PIA) plates. Cultured plates were incubated at 37^0C for 24 hours, and then at 22^0C for 72 hours. Resultant plate phenotypes were scored by eye using ordinal categories 1-4. Mucoidy phenotypes were not included in analysis as they all had the same score. Pyocyanin and pyoverdine production was measured as follows; strains were inoculated into 1.4 mL of Kings A (KA) both and incubated at 37^0C for 24 hours. After 24 hours of growth culture was diluted 1 in 3 in KA, and 1.5 mL of the diluted culture was pelleted in an eppendorf tube so that only 500 uL of culture remained. To measure pyocyanin production, OD readings of this diluted, pelleted culture were taken at 695 nm and 600 nm. Pyocynanin production

was the ratio of 695:600. To measure pyoverdine production, OD readings of this diluted, pelleted culture were taken at 380 nm and 600 nm. Pyocynanin production was the ratio of 380:600.

5.3.7. Fluctuation test

To quantify the mutation rates of the founding strains, and to identify differences in mutation rate at selected ciprofloxacin-resistance conferring genes, we performed a fluctuation test. Single colonies of each founding strain were grown overnight in LB, and then diluted such that approximately 500 cells inoculated each of 96-wells per founding strain. Strains were then grown in 96-well plates in LB for 24 hours. All populations were then streaked onto agar plates containing LB+ciprofloxacin. After 24 hours of growth the number of resistant colonies were recorded and a single colony from each population (if present) was frozen for sequencing. Sanger sequencing was performed by Genome Quebec across five genes (*gyrA, gyrB, nfxB, parC,* and *parE*). Chromatograms were aligned to their respective founding sequence at each gene, and checked visually with CodonCode Aligner (www.codoncode.com). Mutation rates were calculated using the Fluctuation Analysis Calculator (Hall et al. 2009), and 3 different computational methods were used; MSS-Maximum Likelihood Estimator Method (MSS-MLE; Ma et al. 1992; Sarkar et al. 1992), the Lea Coulson Method of the Median (Lea and Coulson, 1949) and a frequency method. All methods gave similar distributions (data not shown) and therefore we proceeded with MSS-MLE mutation rate for analysis. The units of mutation

rate for the MSS-MLE method are mutations per average cell count across cultures (m/Nt).

All statistical analyses were performed in R (2013).

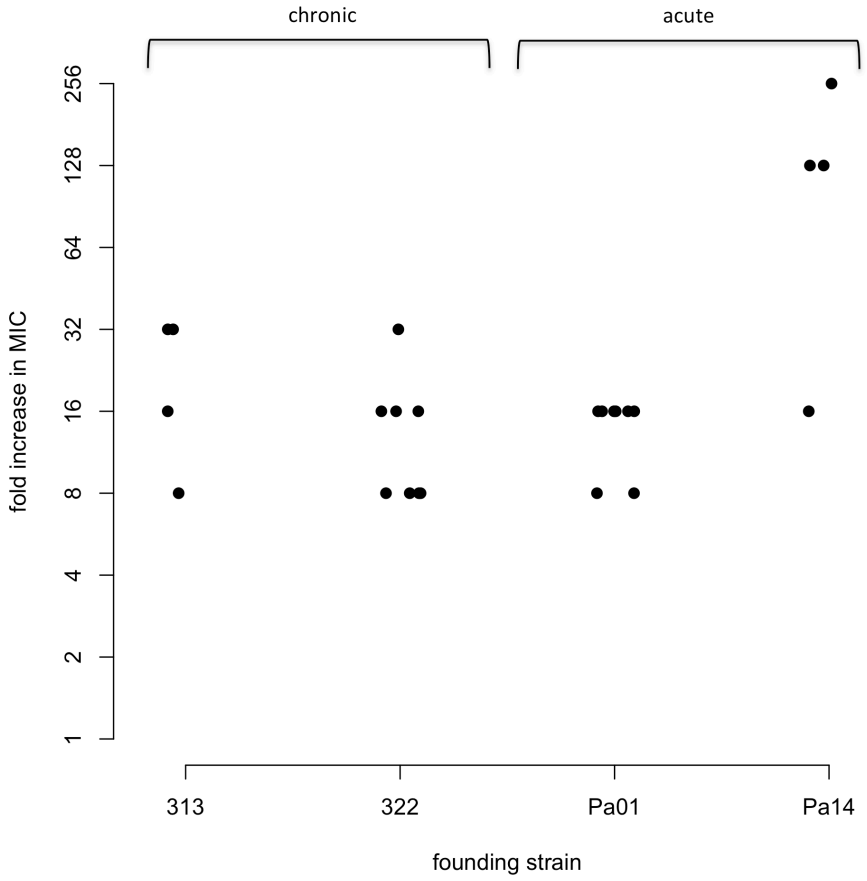

Figure 5.1. Ciprofloxacin resistance in evolved isolates, measured as fold-increase in MIC compared to the respective founding strain. MIC was calculated as the median of two trials for each experimentally evolved isolate.

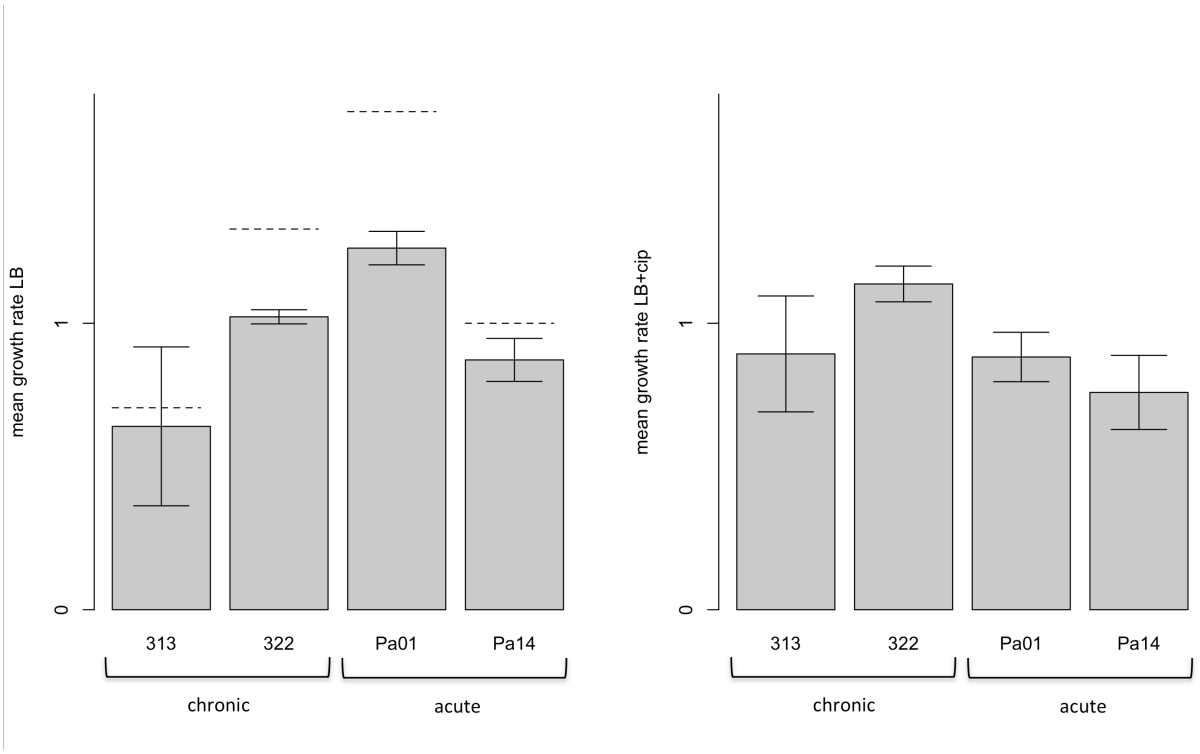

Figure 5.2. Adaptive response of evolved isolates, as measured by relative growth rate in (A) the absence, and (B) the presence of ciprofloxacin. Dashed lines in (A) represent founding strain growth rate in LB. Mean growth rate was calculated from three replicate measures for each experimentally evolved isolate. Error bars represent +/- 1SE.

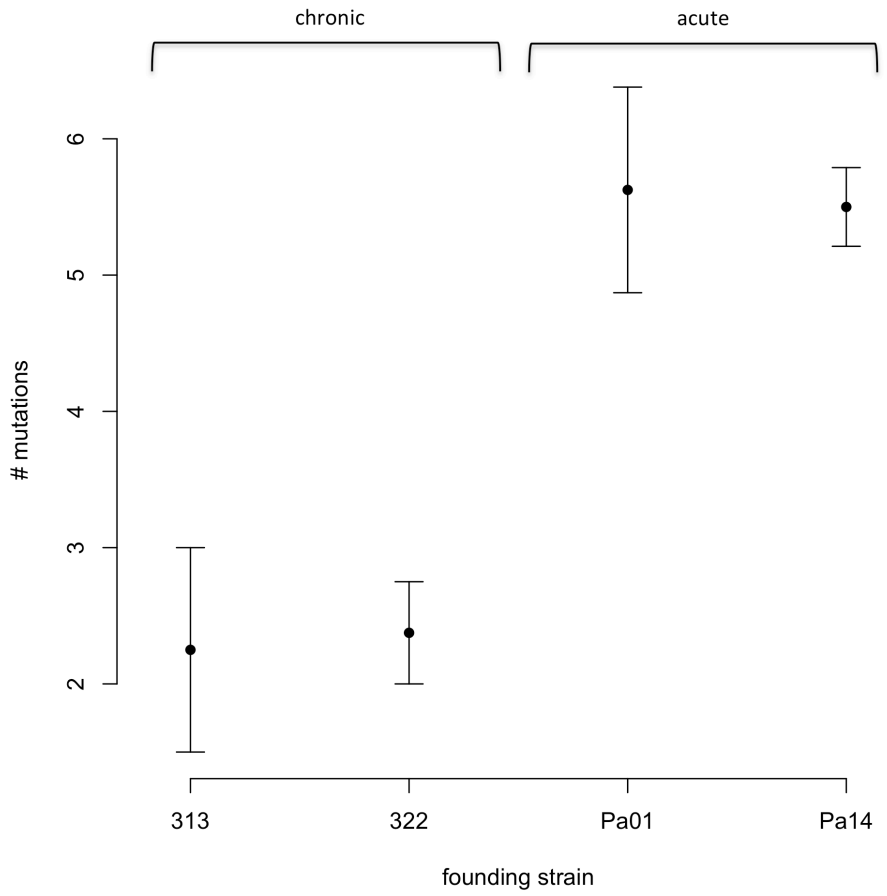

Figure 5.3. Mean number of mutations identified in each evolved isolate, grouped by founding strain. Error bars represent +/- 1SE.

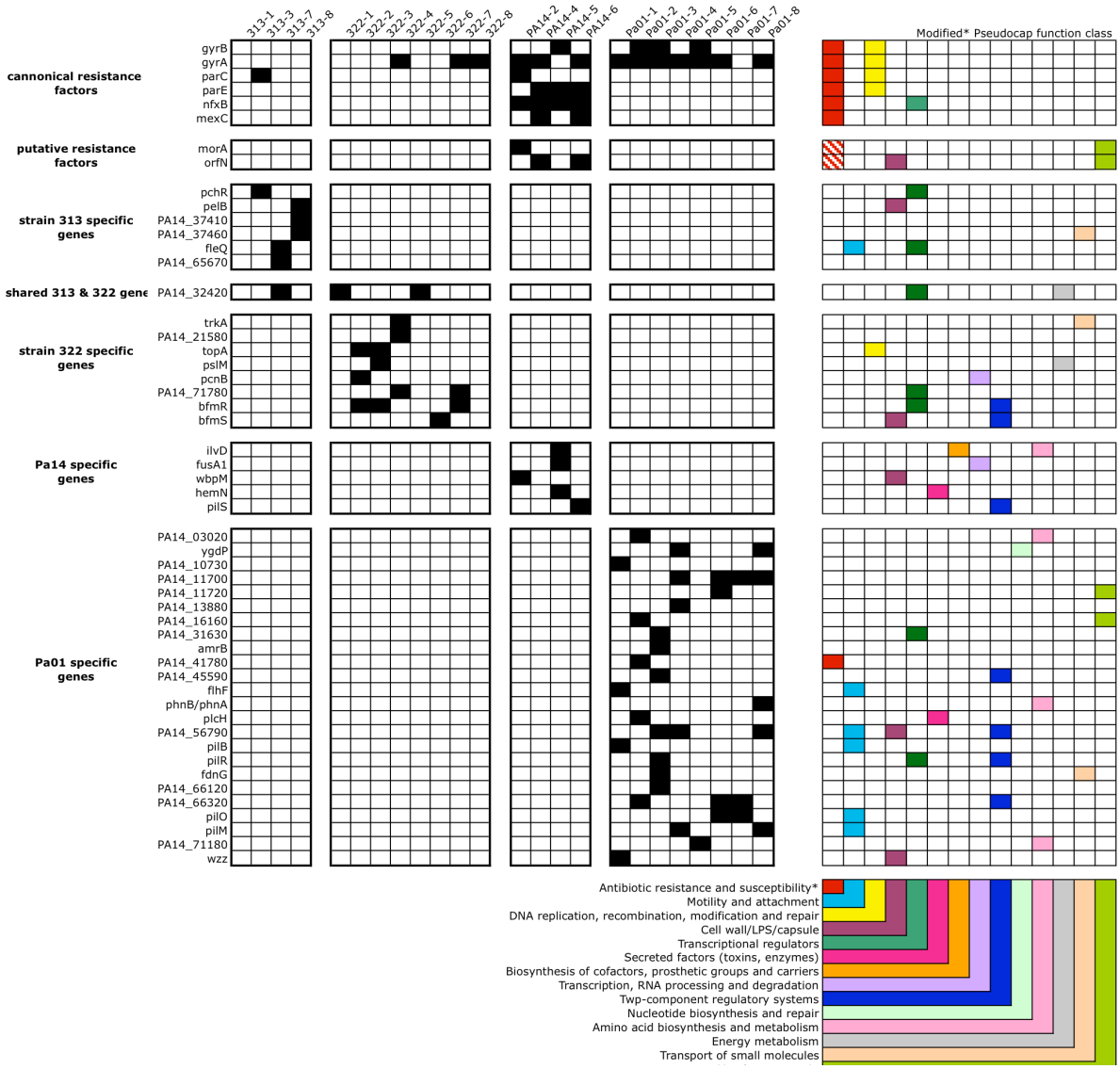

Figure 5.4. Genes containing mutations (n=52) after selection for 200 generations in ciprofloxacin. The black squares in the large matrix denote genes with mutations. The coloured squares in the smaller matrices to the right and below the matrix of amino acid changes denote PseudoCap (Winsor et al. 2011) gene functions.

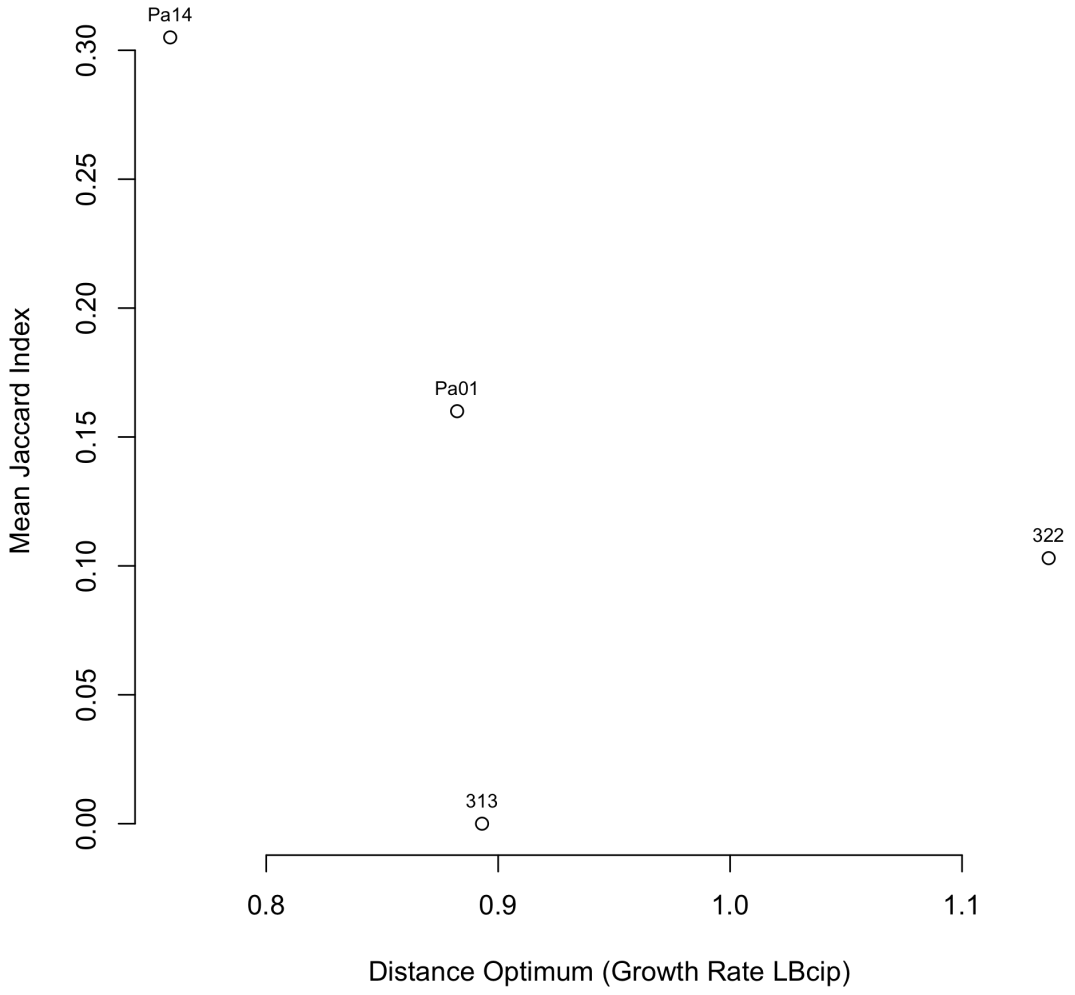

Figure 5.5. Probability of parallel evolution within starting strain as a function of distance to the optimum, measured as growth rate in the presence of antibiotic. Parallel evolution was quantified as the mean Jaccard index. Mean growth rate is the average of three trials.

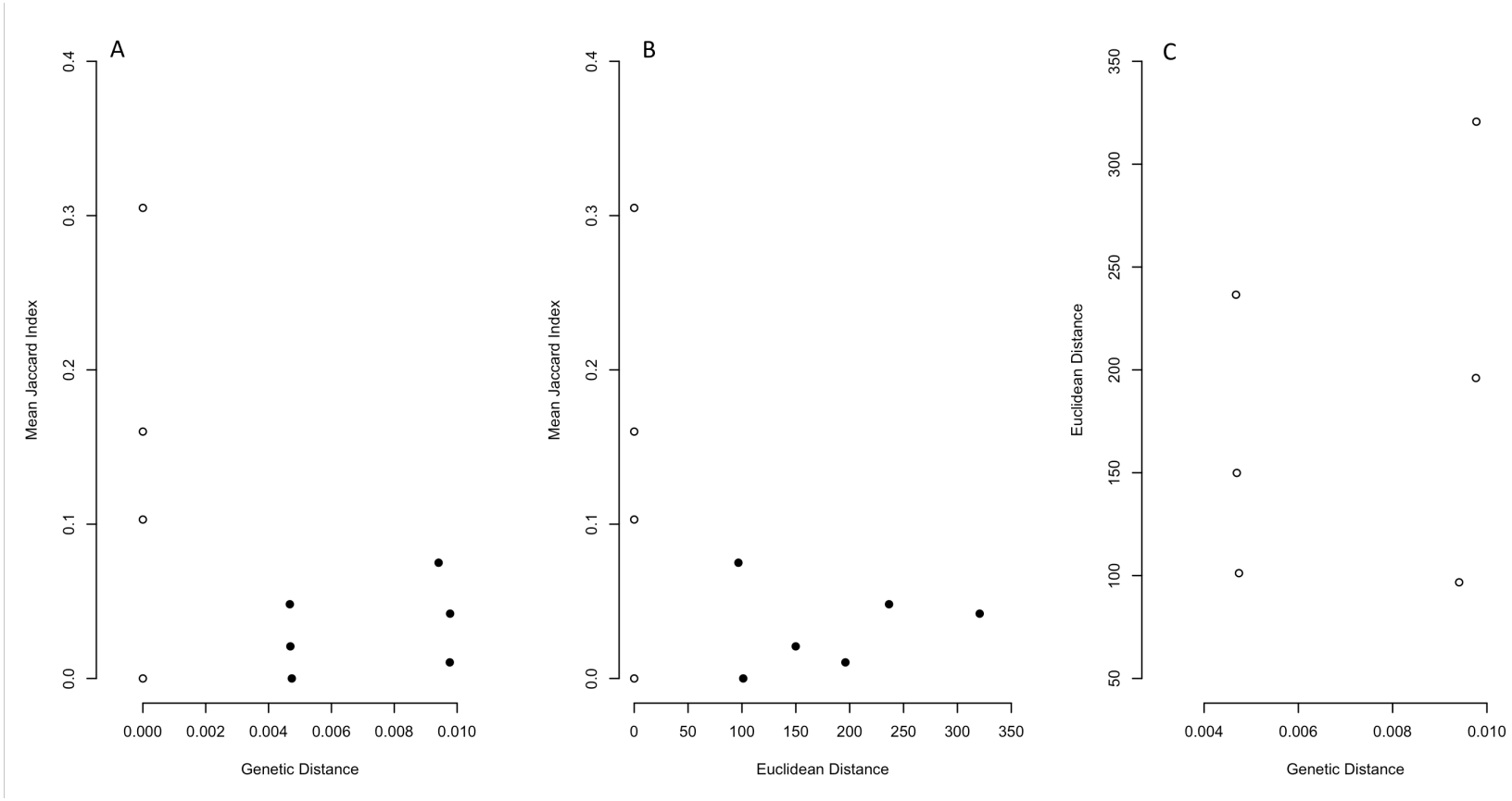

Figure 5.6. Probability of parallel evolution as a function of (A) genetic distance between strains, or (B) Euclidean distance between strains as measured by 10 traits. (C) Genetic distance as a function of Euclidean distance. Filled circles are measures between strains and open circles are measures within a strain.

Chapter 6

General Conclusions

The fitness costs associated with antibiotic resistance mutations will determine how resistance spreads within pathogenic populations. For this thesis I focussed on understanding the process of resistance acquisition and persistence by gaining knowledge about the spectrum of fitness effects associated with antibiotic resistance mutations, using primarily experimental evolution of the opportunistic pathogen *P. aeruginosa*, adapting to the fluoroquinolone antibiotic ciprofloxacin.

In the first chapter of my thesis I showed, using a meta-analysis, that resistance mutations were generally costly, across a range of antibiotics and bacterial species. However, several drug classes and specie of bacteria on average did not show a cost.

Using experimental evolution I have shown that fluctuating drug treatment leads to the evolution of cost-free resistance, whereas resistance is costly in a constant drug treatment. Mutations that conferred resistance were in known genes and shared across both drug treatments. I concluded that cost-free resistance was the result of additional second-site mutations in the fluctuating treatment. Isolating individual resistance mutations from this experiment, I have shown that the mutations conferring ciprofloxacin resistance in *P. aeruginosa* are generally costly. However, costs of resistance were different between ciprofloxacin resistance targets: mutations in efflux pump regulators were significantly more costly than mutations in the drug target, DNA gyrase. Combinations of resistance mutations that evolved together experimentally significantly increased resistance relative to single

resistance mutations, but did not generally exhibit significant epistasis for fitness. The lack of epistatic interactions was a surprising result and indicates that epistasis among evolved antibiotic resistance mutations may not be as widespread as originally thought.

The final chapter of my thesis investigated the repeatability of the genetic causes of ciprofloxacin resistance using experimental evolution of different clinical strains of *P. aeruginosa*. Surprisingly, there was little parallel evolution between strains, and in some cases, resistant evolved isolates did not exhibit any mutations in known resistance-conferring genes. We show that the lack of parallel evolution may be partially explained by differential mutation rates between strains at these known resistance-conferring genes. These results indicate that by using a single representative pathogenic strain to make conclusions for a species as a whole, we may be vastly underestimating the spectrum of antibiotic resistance mutations present in clinical settings.

There are several future lines of investigation that directly stem from this thesis. In chapter three we hypothesized that cost-free resistance was the result of lower cost resistance mutations fixing in the fluctuating treatment. In order to directly test this hypothesis we will construct allelic constructs of each individual resistance mutation and assess their resistance and fitness effects. In chapter four we constructed realized evolutionary combinations of mutations in known ciprofloxacin-resistance conferring genes and tested for epistatic interactions. In order to test the hypothesis that epistatic interactions will differ based on the evolutionary nature of mutational combinations, future work will construct all pair-wise combinations of each of these resistance-conferring mutations, and test for epistatic interactions. In chapter five we showed that novel resistance genes conferred resistance to ciprofloxacin. To ascertain exactly which mutations and genes confer resistance we will make allelic constructs of each individual mutation in evolved isolates for which there was

no known resistance-conferring mutation. Additionally, we will use the allelic constructs of resistance mutations that arose in the PA14 genetic background discussed in chapter four (Table C.1), and place them into the genetic backgrounds of the strains used in chapter five; PA01, JD313 and JD322. Using these constructs we will test for epistatic interactions among known resistance-conferring mutations across different genetic backgrounds.

Here I have used a meta-analysis and two selection experiments to make conclusions about the spectrum of fitness effects associated with antibiotic resistance mutations, and more broadly the evolution of resistance in pathogenic bacterial populations. I have shown that the naïve strategy of halting the use of antibiotics to stop the spread of resistance is unlikely to have the expected results. This is because some resistance mutations are not costly, and if there are costs associated with resistance, these costs are overcome rapidly by second-site mutations. This, along with the demonstration that different strains gain resistance via mutations in a wide range of genes, shows that there are a variety of genetic routes to resistance, and, ultimately, combating the spread of resistance in the future may be even more difficult than anticipated. This work shows the importance of understanding the diverse evolutionary trajectories that underlie the acquisition of antibiotic resistance, and illustrates that this type of knowledge is crucial for managing antibiotic resistance in the future. Thus my thesis represents a valuable contribution to this growing body of work.

Appendix A

Appendix to Chapter 2

A.1 Supplemental Figures and Tables

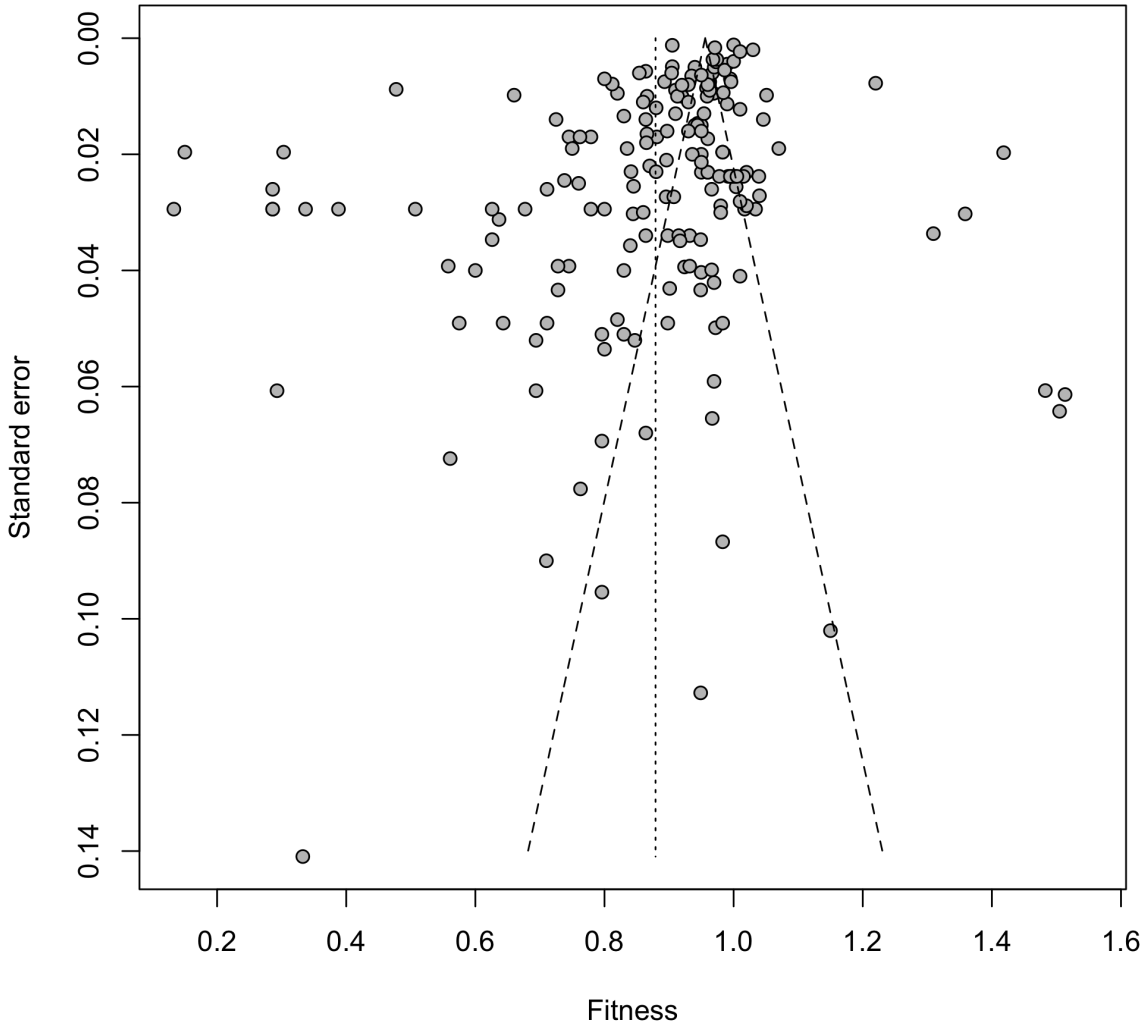

Figure A.1. Funnel plot of mean relative fitness plotted against standard error.

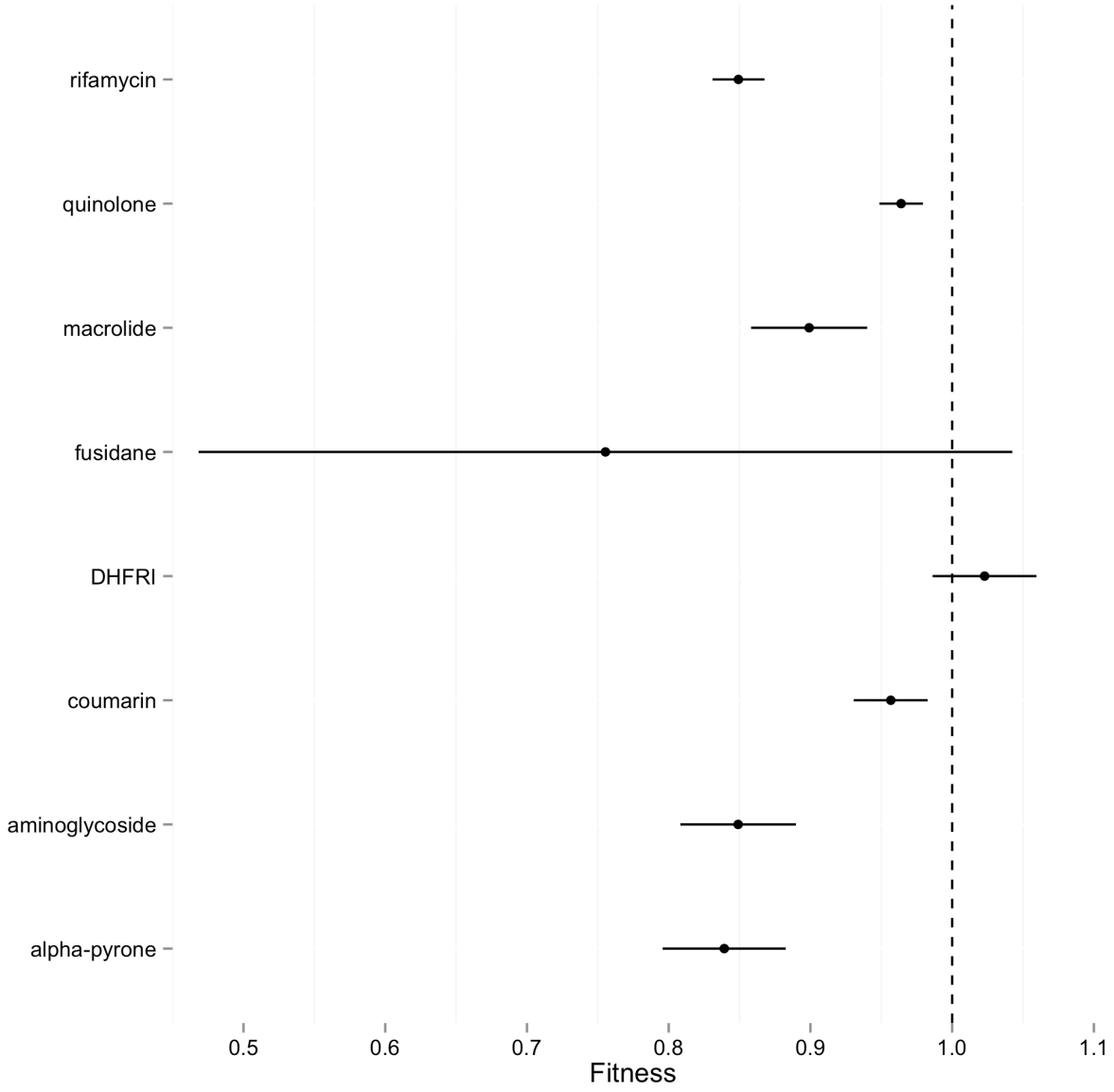

Figure A.2. The mean relative fitness and 95% confidence intervals of antibiotic resistance mutations associated with a given antibiotic class.

Table A.1. Studies included in analysis, indicating antibiotic used.

Study	No. of mutations	amikacin	ciprofloxacin	clarithromycin	coumermycin	erthryomycin	fusidic acid	myxopyronin	nalidixic acid	norfloxacin	novobiocin	olfloxacin	rifampicin	spectinomycin	streptomycin	trimethorprim	tylosin
Almofti et al. 2011	1					X											
Balsalobre & de la Campa 2008	5		X														
Besier et al. 2005	4						X										
Borrell et al. 2013	9											X	X				
Criswell et al. 2006	2													X			
Enne et al. 2004	9												X				
Gagneux et al. 2006	9												X				
Gillespie et al. 2002	1		X														
Han et al 2009	4					X											
Hao et al. 2009	5																X
Lin	8									X							

dgren et al. 2005												
Marcusson et al. 2009	5		X									
Mariam et al. 2004	3									X		
O'Neill et al. 2006	22									X		
Reynolds 2000	9									X		
Rodriguez-Verdugo et al. 2013	8									X		
Rozen et al. 2007	7		X									
Sander et al. 2002	11	X		X							X	
Schrag & Perrot 1996	2										X	
Srivastava et al. 2012	11					X						
Trinidade et al. 2009	19						X			X	X	

Vickers et al. 2009	4										X
Vickers et al. 2007	7		X			X	X				
Wichelhaus et al. 2002	14								X		

Table A.2. Studies included in analysis, indicating bacterial species studied.

Study	No. of mutations	*B. burgdorferi*	*C. jejuni*	*E. coli*	*E. faecium*	*M. smegmatis*	*M. tuberculosis*	*S. aureus*	*S. pneumoniae*
Almofti et al. 2011	1		X						
Balsalobre & de la Campa 2008	5								X
Borrell et al. 2013	9					X			
Besier et al. 2005	4							X	
Criswell et al. 2006	2	X							
Enne et al. 2004	9				X				
Gagneux et al. 2006	9						X		
Gillespie et al. 2002	1								X
Han et al 2009	4		X						
Hao et al. 2009	5		X						
Lindgren et al. 2005	8			X					
Marcusson et al. 2009	5			X					
Mariam et al. 2004	3						X		
O'Neill et al. 2006	22							X	
Reynold	9			X					

s 2000						
Rodrigue z-Verdugo et al. 2013	8	X				
Rozen et al. 2007	7					X
Sander et al. 2002	11		X			
Schrag & Perrot 1996	2	X				
Srivastav a et al. 2012	11				X	
Trinidad e et al. 2009	19	X				
Vickers et al. 2009	4				X	
Vickers et al. 2007	7				X	
Wichelh aus et al. 2002	14				X	

Table A.3. Studies used for subgroup analyses by bacterial species and drug class.

Study	No. of mutations	*S. aureus* quinolone	*S. aureus* rifamycin	*E. coli* quinolone	*E. coli* rifamycin
Balsalobre & de la Campa 2008	5			X	
Lindgren et al. 2005	8			X	
O'Neill et al. 2006	22		X		
Rodriguez-Verdugo et al. 2013	8			X	
Sander et al. 2002	9				X
Trinidade et al. 2009	14				X
Vickers et al. 2007	4	X			
Wichelhaus et al. 2002	14		X		

Appendix B

Appendix to Chapter 3

B.1 Supplemental Table

Table B.1. Mutations detected by Illumina sequencing of evolved isolates. "Sel env" gives the selection treatment as c for constant ciprofloxacin and f2 for fluctuating ciprofloxacin.

sel env	pop	Position	change	Gene_name	Effect	oldAA/newAA	oldcodon/newcodon	codon#
c	1	2015012	SNP	NC_008463.1:gyrA	NON_SYNONYMOUS_CODING	D/N	Gac/Aac	87
c	1	5428070	SNP	NC_008463.1:nfxB	NON_SYNONYMOUS_CODING	L/P	cTg/cCg	14
c	2	2015012	SNP	NC_008463.1:gyrA	NON_SYNONYMOUS_CODING	D/Y	Gac/Tac	87
c	2	2042699	DEL	NC_008463.1:wbpM	FRAME_SHIFT	-/-	-/-	364
c	2	5428070	SNP	NC_008463.1:nfxB	NON_SYNONYMOUS_CODING	L/P	cTg/cCg	14
c	3	5673	DEL	NC_008463.1:gyrB	CODON_DELETION	QE/-	caggag/-	467
c	3	5236647	DEL	NC_008463.1:pilC	FRAME_SHIFT	-/-	-/-	189
c	3	5428070	SNP	NC_008463.1:nfxB	NON_SYNONYMOUS_CODING	L/P	cTg/cCg	14
c	4	5706	DEL	NC_008463.1:gyrB	CODON_DELETION	CGI/-	tgtggcatc/-	478
c	4	5428070	SNP	NC_008463.1:nfxB	NON_SYNONYMOUS_CODING	L/P	cTg/cCg	14
c	5	5678	SNP	NC_008463.1:gyrB	NON_SYNONYMOUS_CODING	E/D	gaG/gaT	468
c	5	3960421	INS	NC_008463.1:hemN	FRAME_SHIFT	-/?	-/C	283
c	5	5428070	SNP	NC_008463.1:nfxB	NON_SYNONYMOUS_CODING	L/P	cTg/cCg	14
c	6	2015001	SNP	NC_008463.1:gyrA	NON_SYNONYMOUS_CODING	T/I	aCc/aTc	83
c	6	2040296	DEL	NC_008463.1:orfN	FRAME_SHIFT	-/-	-/-	50
c	6	5428070	SNP	NC_008463.1:nfxB	NON_SYNONYMOUS_CODING	L/P	cTg/cCg	14
c	7	5683	SNP	NC_008463.1:gyrB	NON_SYNONYMOUS_CODING	G/D	gGt/gAt	470
c	7	2043095	DEL	NC_008463.1:wbpM	FRAME_SHIFT	-/-	-/-	496
c	7	5428144	SNP	NC_008463.1:nfxB	NON_SYNONYMOUS_CODING	T/P	Acc/Ccc	39
c	8	2015012	SNP	NC_008463.1:gyrA	NON_SYNONYMOUS_CODING	D/Y	Gac/Tac	87
c	8	5428070	SNP	NC_008463.1:nfxB	NON_SYNONYMOUS_CODING	L/P	cTg/cCg	14
c	8	5432300	SNP	NC_008463.1:morA	NON_SYNONYMOUS_CODING	L/Q	cTg/cAg	1155
f2	1	2016597	SNP	NC_008463.1:gyrA	NON_SYNONYMOUS_CODING	A/E	gCg/gAg	615
f2	1	5428091	SNP	NC_008463.1:nfxB	NON_SYNONYMOUS_CODING	H/P	cAc/cCc	21
f2	1	5432189	SNP	NC_008463.1:morA	NON_SYNONYMOUS_CODING	L/Q	cTg/cAg	1118
f2	2	1858766	DEL	GI:116051309	FRAME_SHIFT	-/-	-/-	65
f2	2	5428091	SNP	NC_008463.1:nfxB	NON_SYNONYMOUS_CODING	H/P	cAc/cCc	21
f2	2	5431676	SNP	NC_008463.1:morA	NON_SYNONYMOUS_CODING	V/A	gTg/gCg	947
f2	3	1407080	SNP	NC_008463.1:wspA	NON_SYNONYMOUS_CODING	P/L	cCg/cTg	479
f2	3	2015012	SNP	NC_008463.1:gyrA	NON_SYNONYMOUS_CODING	D/N	Gac/Aac	87
f2	3	5428252	SNP	NC_008463.1:nfxB	STOP_GAINED	E/*	Gag/Tag	75
f2	4	532305	DEL	GI:116054188	FRAME_SHIFT	-/-	-/-	404
f2	4	1406645	SNP	NC_008463.1:wspA	NON_SYNONYMOUS_CODING	A/V	gCc/gTc	334
f2	4	2015013	SNP	NC_008463.1:gyrA	NON_SYNONYMOUS_CODING	D/G	gAc/gGc	87
f2	4	5427823	SNP	NC_008463.1:mexC	NON_SYNONYMOUS_CODING	A/V	gCc/gTc	16
f2	4	5428252	SNP	NC_008463.1:nfxB	STOP_GAINED	E/*	Gag/Tag	75
f2	5	5670	SNP	NC_008463.1:gyrB	NON_SYNONYMOUS_CODING	S/T	Tcc/Acc	466
f2	5	2015012	SNP	NC_008463.1:gyrA	NON_SYNONYMOUS_CODING	D/Y	Gac/Tac	87
f2	5	5428144	SNP	NC_008463.1:nfxB	NON_SYNONYMOUS_CODING	T/P	Acc/Ccc	39
f2	6	1412896	DEL	NC_008463.1:wspF	FRAME_SHIFT	-/-	-/-	285
f2	6	2015001	SNP	NC_008463.1:gyrA	NON_SYNONYMOUS_CODING	T/I	aCc/aTc	83
f2	6	2040296	DEL	NC_008463.1:orfN	FRAME_SHIFT	-/-	-/-	50
f2	6	5423304	DEL	NC_008463.1:oprJ	FRAME_SHIFT	-/-	-/-	76
f2	6	5428511	DEL	NC_008463.1:nfxB	FRAME_SHIFT	-/-	-/-	161
f2	8	2015013	SNP	NC_008463.1:gyrA	NON_SYNONYMOUS_CODING	D/G	gAc/gGc	87
f2	8	5428070	SNP	NC_008463.1:nfxB	NON_SYNONYMOUS_CODING	L/P	cTg/cCg	14
f2	8	5432482	INS	NC_008463.1:morA	CODON_INSERTION	-/G	-/GGC	1216

Appendix C

Appendix to Chapter 4

C.1 Supplemental Table

Table C.1. Fitness and resistance of single and double-resistant allelic constructs.

Gene	Allele	Median Fold-increase in MIC	Mean Fitness	Standard Error of Mean Fitness
gyrA	D87N	4	1.009	0.0125
nfxB	L14P	4	0.818	0.0249
gyrA+nfxB	D87N+L14P	32	0.972	0.0841
gyrA	D87Y	6	1.018	0.00803
gyrA+nfxB	D87Y+L14P	32	0.818	0.0193
gyrB	QE467-	2	1.011	0.0117
gyrB+nfxB	QE467-+L14P	16	0.909	0.0168
gyrB	CGI478-	1	0.957	0.0127
gyrB+nfxB	CGI478-+L14P	8	0.847	0.0455
gyrB	E468D	1.25	1.031	0.0136
gyrB+nfxB	E468D+L14P	16	0.837	0.0157
gyrA	T83I	8	0.847	0.0318
gyrA+nfxB	T83I+L14P	64	0.888	0.0135

gyrB	G470D	1	0.988	0.0176
nfxB	T39P	4	0.833	0.00839
gyrB+*nfxB*	G470D+T39P	12	0.858	NA
nfxB	E75*	4	0.929	0.0220
gyrA+*nfxB*	D87N+E75*	32	0.925	0.0233
gyrA	D87G	4	1.008	0.00583
gyrA+*nfxB*	D87G+E75*	16	0.958	0.0322
gyrB	S466T	1.5	1.028	0.0149
gyrA+*gyrB*	D87Y+S466T	10	1.020	0.0332
nfxB	fs161	4	0.870	0.0309
gyrA+*nfxB*	T83I+fs161	64	0.943	0.0802
gyrA+*nfxB*	D87G+L14P	16	0.848	0.0475

Appendix D

Appendix to Chapter 5

D.1 Supplemental Figures and Tables

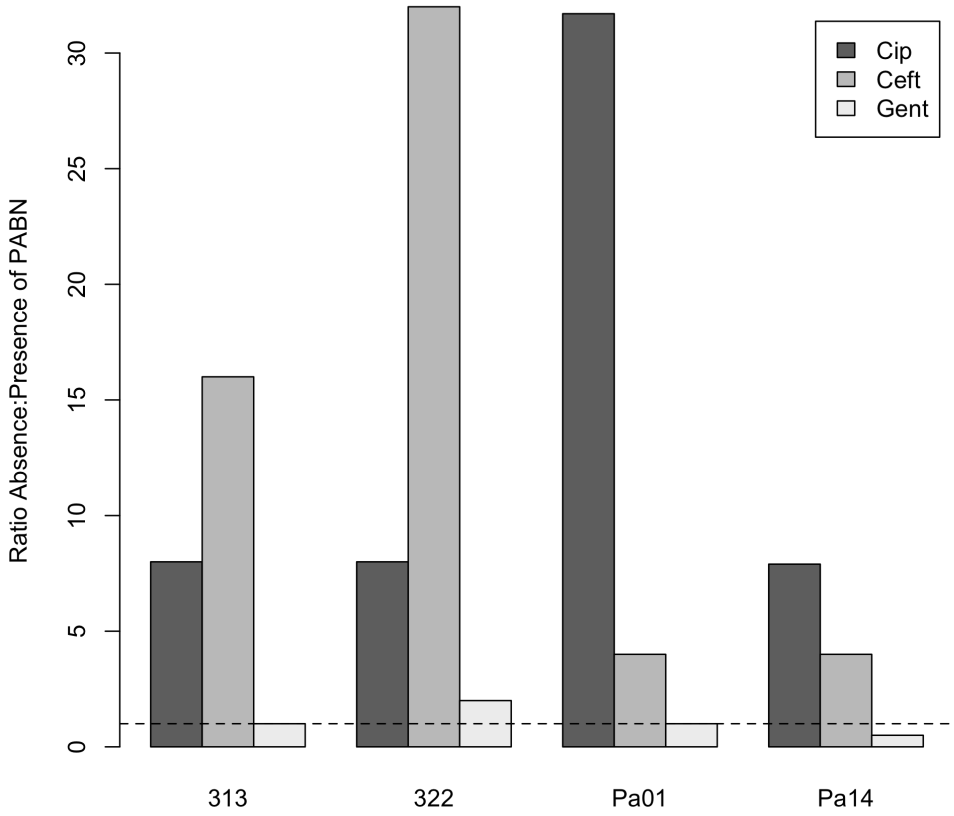

Figure D.1. The ratio of MICs in the absence to presence of PAbN for three different antibiotics; Cip – ciprofloxacin, Ceft – ceftazidime, Gent – gentamycin. The dashed line represents a 1:1 ratio.

Table D.1. Tukey test for differences in evolved resistance (MIC) between founding strains.

Strains	p-value
322-313	0.692
Pa01-313	0.814
Pa14-313	**0.013**
Pa01-322	0.993
Pa14-322	**<0.001**
Pa14-Pa01	**<0.001**

Table D.2. Tukey test for differences in ancestral and evolved growth rate in LB.

Strains	Ancestral p-value	Evolved p-value
322-313	**<0.0001**	**0.006**
Pa01-313	**<0.0001**	**<0.001**
Pa14-313	**<0.0001**	0.095
Pa01-322	**<0.0001**	0.527
Pa14-322	**<0.0001**	0.792
Pa14-Pa01	**<0.0001**	0.204

Table D.3. Growth rate in LB decreased through time for most founding strains.

Strain	Mean ancestral growth rate LB (+/-SE)	Mean evolved growth rate LB (+/-SE)	t-value	p-value
Pa14	1.00 (0.084)	0.872 (0.075)	1.38	0.184
Pa01	**1.74 (0.015)**	**1.26 (0.058)**	**10.3**	**<0.0001**
313	**0.793 (0.092)**	**0.640 (0.277)**	**2.26**	**0.039**
334	**1.33 (0.007)**	**1.02 (0.025)**	**16.0**	**<0.0001**

Table D.4. The fate of evolved populations in this experiment. Note that only isolates that have a mutation number listed (in bold) are included in analysis (mutator, contaminated populations and populations for which there were sequencing errors have been excluded from further analyses).

Ancestor	Evolved Population	# mutations identified
Pa14	1	NA (contaminated)
Pa14	**2**	**5 mutations**
Pa14	3	NA (contaminated)
Pa14	**4**	**5 mutations**
Pa14	**5**	**6 mutations**
Pa14	**6**	**6 mutations**
Pa14	7	NA (contaminated)
Pa14	8	mutator
Pa01	**1**	**5 mutations**
Pa01	**2**	**8 mutations**
Pa01	**3**	**9 mutations**
Pa01	**4**	**6 mutations**
Pa01	**5**	**3 mutations**
Pa01	**6**	**5 mutations**
Pa01	**7**	**3 mutations**
Pa01	**8**	**6 mutations**
JD313	**1**	**0 mutations**
JD313	2	mutator

JD313	**3**	**3 mutations**
JD313	4	Poor sequencing quality
JD313	5	mutator
JD313	6	mutator
JD313	**7**	**3 mutations**
JD313	**8**	**3 mutations**
JD322	**1**	**1 mutation**
JD322	**2**	**3 mutations**
JD322	**3**	**3 mutations**
JD322	**4**	**4 mutations**
JD322	**5**	**1 mutation**
JD322	**6**	**2 mutations**
JD322	**7**	**3 mutations**
JD322	**8**	**2 mutations**
JD334	1	mutator
JD334	2	NA (contaminated)
JD334	3	NA (contaminated)
JD334	4	NA (contaminated)
JD334	5	mutator
JD334	6	Not quantified
JD334	7	mutator

Table D.5. Mutations detected by Illumina sequencing of evolved isolates. “Strain” gives the founding strain, and all locations are relative to the PA14 reference genome number NC_008463.1, unless yellow, in which case the gene is not present in the PA14 reference genome and the coordinates refer to the PA01 reference genome NC_002516.

strain	pop	position	change	Gene_name	Effect	oldAA/newAA	oldcodon/newcodon	codon_num
313	3	785454	SNP	pchR	SYNONYMOUS_CODING	P/P	ccG/ccC	277
313	3	5845615	SNP	parC	NON_SYNONYMOUS_CODING	A/P	Gcc/Ccc	88
313	3	5464967	SNP		INTERGENIC			
313	7	2820758	SNP	PA14_32420	NON_SYNONYMOUS_CODING	H/L	cAc/cTc	262
313	7	4461490	SNP	fleQ	SYNONYMOUS_CODING	A/A	gcG/gcC	285
313	7	5849456	SNP	PA14_65670	STOP_GAINED	R/*	Cga/Tga	162
313	8	2139955	DEL	pelB	FRAME_SHIFT	-/-	-/-	555
313	8	3330177	INS	PA14_37410	FRAME_SHIFT	-/?	-/CC	141
313	8	3333372	DEL	PA14_37460	FRAME_SHIFT	-/-	-/-	421
322	1	2820901	DEL	PA14_32420	FRAME_SHIFT	-/-	-/-	310
322	2	2197362	DEL	topA	CODON_DELETION	NVRF/-	aacgtgcgcttc/-	238
322	2	5582384	DEL	pcnB	FRAME_SHIFT	-/-	-/-	377
322	2	4585842	DEL	bfmR	CODON_DELETION			
322	3	2198500	SNP	topA	NON_SYNONYMOUS_CODING	G/A	gGc/gCc	617
322	3	3163050	SNP	pslM	NON_SYNONYMOUS_CODING	M/R	aTg/aGg	169
322	3	4585252	DEL	bfmR	FRAME_SHIFT			
322	4	18316	DEL	trkA	CODON_DELETION	VIDFAE/-	gtgatcgacttcgccgaa/-	144
322	4	1871079	DEL	PA14_21580	FRAME_SHIFT	-/-	-/-	202
322	4	2015001	SNP	gyrA	NON_SYNONYMOUS_CODING	T/I	aCc/aTc	83
322	4	6394532	SNP	PA14_71780	STOP_GAINED	K/*	Aag/Tag	149
322	5	2820720	DEL	PA14_32420	CODON_CHANGE_PLUS_CODON_DELETION	NESA/K	aacgagtcggcg/aag	249
322	6	5907427	SNP		INTERGENIC			
322	6	4587146	INS	bfmS	FRAME_SHIFT			
322	7	2015001	SNP	gyrA	NON_SYNONYMOUS_CODING	T/I	aCc/aTc	83
322	7	6394532	SNP	PA14_71780	STOP_GAINED	K/*	Aag/Tag	149
322	7	4585647	DEL	bfmR	CODON_DELETION			
322	8	2015012	SNP	gyrA	NON_SYNONYMOUS_CODING	D/Y	Gac/Tac	87
322	8	5907427	SNP		INTERGENIC			
PA01	1	924321	INS	PA14_10730	FRAME_SHIFT	-/A?	-/GCAT	344
PA01	1	2016013	SNP	gyrA	SYNONYMOUS_CODING	A/A	gcG/gcC	420
PA01	1	4061075	INS	flhF	CODON_INSERTION	D/DA	gac/gaCGCc	204
PA01	1	5234491	DEL	pilB	CODON_DELETION	SLERD/S	agccttgagcgggac/agc	71
PA01	1	3546696	DEL	wzz	CODON_DELETION			272
PA01	2	6379	DEL	gyrB	CODON_CHANGE_PLUS_CODON_DELETION	LLEDG/R	ctgctggaagacggc/cgc	702
PA01	2	136654	DEL		INTERGENIC			
PA01	2	267076	SNP	PA14_03020	NON_SYNONYMOUS_CODING	T/P	Acc/Ccc	21
PA01	2	1376401	INS	PA14_16160	FRAME_SHIFT	-/G?	-/GGCC	139
PA01	2	2015968	SNP	gyrA	SYNONYMOUS_CODING	A/A	gcC/gcC	405
PA01	2	3728989	DEL	PA14_41780	CODON_DELETION	FPQI/-	tttccgcagatc/-	227
PA01	2	4731270	DEL	plcH	CODON_CHANGE_PLUS_CODON_DELETION	PWKDG/R	ccgtggaaggatggc/cgc	372
PA01	2	5914408	INS	PA14_66320	CODON_INSERTION	-/LR	-/CTGCGC	668
PA01	3	6353	DEL	gyrB	CODON_CHANGE_PLUS_CODON_DELETION	SLLGDQLN/S	tcqctqctcqqcqaccaqctqaac/tcc	693
PA01	3	2015968	SNP	gyrA	SYNONYMOUS_CODING	A/A	gcC/gcC	405
PA01	3	2748559	DEL	PA14_31630	FRAME_SHIFT	-/-	-/-	271
PA01	3	3423054	DEL	amrB	FRAME_SHIFT	-/-	-/-	55
PA01	3	4056137	DEL	PA14_45590	FRAME_SHIFT	-/-	-/-	349
PA01	3	5058992	INS	PA14_56790	FRAME_SHIFT	-/A?	-/GCGC	328
PA01	3	5367913	DEL	pilR	FRAME_SHIFT	-/-	-/-	154
PA01	3	5670029	DEL	fdnG	FRAME_SHIFT	-/-	-/-	770
PA01	3	5890851	DEL	PA14_66120	CODON_CHANGE_PLUS_CODON_DELETION	AHCEC/G	gcccattgcgagtgc/ggc	24
PA01	4	392497	INS	ygdP	CODON_CHANGE_PLUS_CODON_INSERTION	Y/CY	tac/tGCTac	77
PA01	4	1014497	SNP	PA14_11700	SYNONYMOUS_CODING	A/A	gCc/gCc	38
PA01	4	1193075	SNP	PA14_13880	NON_SYNONYMOUS_CODING	S/R	Agc/Cgc	22
PA01	4	2016012	SNP	gyrA	NON_SYNONYMOUS_CODING	A/V	gCg/gTg	420
PA01	4	5059299	DEL	PA14_56790	CODON_CHANGE_PLUS_CODON_DELETION	RDALQ/Q	cgcgacgccctgcag/cag	430
PA01	4	5951841	INS	pilM	FRAME_SHIFT	-/?	-/C	88
PA01	5	6353	DEL	gyrB	CODON_CHANGE_PLUS_CODON_DELETION	SLLGDQLN/S	tcqctqctcqqcqaccaqctqaac/tcc	693
PA01	5	2015968	SNP	gyrA	SYNONYMOUS_CODING	A/A	gcC/gcC	405
PA01	5	6341164	DEL	PA14_71180	FRAME_SHIFT	-/-	-/-	281
PA01	6	1014497	SNP	PA14_11700	SYNONYMOUS_CODING	A/A	gCc/gCc	38
PA01	6	1014765	SNP	PA14_11720	SYNONYMOUS_CODING	G/G	ggC/ggT	171
PA01	6	2016012	SNP	gyrA	NON_SYNONYMOUS_CODING	A/V	gCg/gTg	420
PA01	6	5914927	INS	PA14_66320	FRAME_SHIFT	-/*?	-/TGATC	841
PA01	6	5949876	DEL	pilO	CODON_CHANGE_PLUS_CODON_DELETION	RM/L	cgcatg/ctg	190
PA01	7	1014497	SNP	PA14_11700	SYNONYMOUS_CODING	A/A	gCc/gCc	38
PA01	7	5914927	INS	PA14_66320	FRAME_SHIFT	-/*?	-/TGATC	841
PA01	7	5949876	DEL	pilO	CODON_CHANGE_PLUS_CODON_DELETION	RM/L	cgcatg/ctg	190
PA01	8	392497	INS	ygdP	CODON_CHANGE_PLUS_CODON_INSERTION	Y/CY	tac/tGCTac	77
PA01	8	1014497	SNP	PA14_11700	SYNONYMOUS_CODING	A/A	gCc/gCc	38
PA01	8	2016012	SNP	gyrA	NON_SYNONYMOUS_CODING	A/V	gCg/gTg	420
PA01	8	4564377	DEL	phnB/phnA	FRAME_SHIFT	-/-	-/-	5 / 528
PA01	8	5059299	DEL	PA14_56790	CODON_CHANGE_PLUS_CODON_DELETION	RDALQ/Q	cgcgacgccctgcag/cag	430
PA01	8	5951841	INS	pilM	FRAME_SHIFT	-/?	-/C	88
PA14	2	2015012	SNP	gyrA	NON_SYNONYMOUS_CODING	D/Y	Gac/Tac	87
PA14	2	2042699	DEL	wbpM	FRAME_SHIFT	-/-	-/-	364
PA14	2	5428070	SNP	nfxB	NON_SYNONYMOUS_CODING	L/P	cTg/cCg	14
PA14	2	5432161	SNP	morA	NON_SYNONYMOUS_CODING	A/T	Gcc/Acc	1109
PA14	2	5845329	SNP	parC	NON_SYNONYMOUS_CODING	M/T	aTg/aCg	183
PA14	4	2015001	SNP	gyrA	NON_SYNONYMOUS_CODING	T/I	aCc/aTc	83
PA14	4	2040296	DEL	orfN	FRAME_SHIFT	-/-	-/-	50
PA14	4	5426812	SNP	mexC	NON_SYNONYMOUS_CODING	D/G	gAc/gGc	353
PA14	4	5428070	SNP	nfxB	NON_SYNONYMOUS_CODING	L/P	cTg/cCg	14
PA14	4	5847897	SNP	parE	NON_SYNONYMOUS_CODING	P/S	Ccg/Tcg	471
PA14	5	5678	SNP	gyrB	NON_SYNONYMOUS_CODING	E/D	gaG/gaT	468
PA14	5	410985	SNP	ilvD	NON_SYNONYMOUS_CODING	A/S	Gcc/Tcc	358
PA14	5	755679	SNP	fusA1	SYNONYMOUS_CODING	T/T	acA/acG	5
PA14	5	3959915	DEL	hemN	FRAME_SHIFT	-/-	-/-	114
PA14	5	5428070	SNP	nfxB	NON_SYNONYMOUS_CODING	L/P	cTg/cCg	14
PA14	5	5847931	SNP	parE	NON_SYNONYMOUS_CODING	E/D	gaG/gaT	459
PA14	6	2015001	SNP	gyrA	NON_SYNONYMOUS_CODING	T/I	aCc/aTc	83
PA14	6	2040296	DEL	orfN	FRAME_SHIFT	-/-	-/-	50
PA14	6	5366925	DEL	pilS	CODON_DELETION	L/-	ctg/-	361
PA14	6	5426773	SNP	mexC	NON_SYNONYMOUS_CODING	G/V	gGg/gTg	366
PA14	6	5428070	SNP	nfxB	NON_SYNONYMOUS_CODING	L/P	cTg/cCg	14
PA14	6	5847897	SNP	parE	NON_SYNONYMOUS_CODING	P/S	Ccg/Tcg	471

Table D.6. Tukey test for differences in mean number of mutations between founding strains.

Strains	p-value
322-313	0.999
Pa01-313	**0.009**
Pa14-313	**0.034**
Pa01-322	**0.002**
Pa14-322	**0.017**
Pa14-Pa01	0.999

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