

The genomics of adaptation to mutational degradation in hypermutator *Pseudomonas aeruginosa*

Karl Heilbron

The Queen's College

University of Oxford

Thesis submitted for the degree of *Doctor of Philosophy*

October 9th, 2015

Table of Contents

Acknowledgements.....3

Abstract.....5

Author Contributions.....6

General Introduction.....8

Chapter 1: Fitness is strongly influenced by rare mutations of large effect in a microbial mutation accumulation experiment.....13

Chapter 2: The genomics of adaptation to mutational degradation in hypermutator *Pseudomonas aeruginosa*.....44

Chapter 3: Adaptation to a novel environment can decrease the cost of rifampicin resistance in *Pseudomonas aeruginosa*.....68

General Discussion 80

Appendix: The genomic basis of adaptation to the fitness cost of rifampicin resistance in *Pseudomonas aeruginosa*.....87

References.....110

Acknowledgements

The 128 pages of this thesis represent the culmination of my academic endeavours over the past four years. The words herein, however, fail to capture many of the most important things that I have learned during my time at Oxford. Absent are the incredible friendships, the long conversations, and the million intangible ways that I have changed as a result.

There are many people who have supported me in my work and I would like to give my sincerest thanks to them now. First and foremost, I would like to thank my supervisor, Craig MacLean for having faith in me and allowing me great freedom in how I have pursued my work, while at the same time providing excellent guidance and direction. Furthermore, the quality of my work has been greatly improved by contributions from my co-authors in the MacLean group—Danna, Macarena, Qi Qin, and Mila—as well as from excellent comments during group meetings from the rest of the MacLean group and our Theoretical and Experimental Evolutionary Biology supergroup.

In particular I would like to acknowledge Alvaro San Millan. Alvaro has never failed to be there for me at the drop of a hat, regardless of how busy he is. He has always given me his full attention and has invested heavily in my work, whether it be celebrating a good result, looking over something I have written, or letting me bounce ideas off of him. I will greatly miss working with Alvaro and I wish him all the best in establishing his own laboratory in Madrid.

I am extremely grateful for the people and organizations that have provided me with funding: The Clarendon Fund, The Queen's College (Oxford), The Natural Sciences and Engineering Research Council of Canada, and Craig MacLean. Not only have you allowed me to pursue a career in evolutionary biology, you have enabled me to attend incredible conferences and courses that have greatly influenced my development as a scientist.

Finally, I would like to dedicate my DPhil thesis to my parents, Louise and Brett

Heilbron. Thank you for all the sacrifices you have made for me. Thank you for working so hard to afford me every opportunity to reach my full potential. Thank you for pushing me to excel, but never pushing me towards a destiny that I had not chosen myself.

Karl Heilbron



September 15th, 2015

Abstract

Genetic diversity is generated and maintained even in the simplest of environments. Because interactions between pairs of mutations are incredibly common, genetic diversity may interact with incoming beneficial mutations and alter the evolutionary paths that are available to different genotypes. In this thesis I have explored how divergent genotypes respond to evolutionary forces like drift and selection with a particular focus on the initial fitness of these genotypes. In order to generate genetically divergent starting populations, in Chapter 1 I performed a mutation accumulation (MA) experiment using a hypermutator strain of *Pseudomonas aeruginosa*. This allowed me to characterize the rate and effect of spontaneous mutations in this system, and I found that fitness is strongly influenced by rare mutations of large effect. In Chapter 2 I evolved several replicates of each of these genetically divergent lines under laboratory conditions and sequenced their genomes to determine the genetic mechanisms underpinning adaptation to mutational degradation. Interestingly, acquiring general laboratory adaptation mutations was more important than specifically compensating for the mutations that occurred during MA. Furthermore, fitness of an MA line prior to recovery played a large role in determining the ruggedness of the adaptive landscape experienced by evolving populations. Given the importance of initial fitness in evolution that I observed in Chapter 2, in Chapter 3 I directly tested whether the initial fitness of a strain could influence the fitness effect of incoming mutations in the context of antibiotic resistance evolution. Adaptation to a novel environment could decrease the cost of antibiotic resistance, but there was no systematic relationship between initial fitness and cost. Taken together, my work has contributed to our understanding of how the fitness of a genotype and the nature of incoming mutations (*i.e.* beneficial or deleterious) interact to influence the outcome of evolution.

Author Contributions

Chapter 1: Heilbron K, Toll-Riera M, Kojadinovic M and MacLean RC (2014) Fitness is strongly influenced by rare mutations of large effect in a microbial mutation accumulation experiment. *Genetics* 197:981-990.

- KH: performed the mutation accumulation experiment, fitness assays, statistical analysis, and simulations, assisted with gDNA extractions, wrote the paper.
- MTR: performed all bioinformatic analyses, statistical tests on those data, wrote the relevant sections of the paper.
- MK: designed and performed gDNA extractions for genome sequencing
- RCM: conceived the experiment, provided feedback at every stage of the experiment, edited the paper.

Chapter 2: Heilbron K, Gifford D and MacLean RC (unpublished) The genomics of adaptation to mutational degradation in hypermutator *Pseudomonas aeruginosa*.

- KH: performed the selection experiment, fitness and motility assays, gDNA extractions, phylogenetic and statistical analysis, simulations, wrote the paper.
- DG: quality control, read mapping, and variant calling of next-generation sequencing data, wrote the relevant sections of the paper.
- RCM: conceived the experiment, provided feedback at every stage of the experiment, edited the paper.

Chapter 3: Heilbron K, Qi Q and MacLean RC (unpublished) Adaptation to a novel environment can decrease the cost of rifampicin resistance in *Pseudomonas aeruginosa*.

- KH: performed fluctuation assays, *rpoB* sequencing, fitness assays, statistical analysis, wrote the paper.

- QQ: contributed strains from a previous experiment, edited the paper.
- RCM: conceived the experiment, provided feedback at every stage of the experiment, edited the paper.

I have also contributed to two other papers during my DPhil. The latter can be found in the Appendix since my work in Chapter 3 follows on from it directly.

- San Millan A, Heilbron K and MacLean RC (2014) Positive epistasis between co-infecting plasmids promotes plasmid survival in bacterial populations. *The ISME Journal* 8:601-612.
- Qi Q, Toll-Riera M, Heilbron K and MacLean RC (submitted to *Molecular Biology and Evolution*) The genomic basis of adaptation to the fitness cost of rifampicin resistance in *Pseudomonas aeruginosa*.

General Introduction

I have been given permission to submit my thesis as a collection of papers by Adrian Thomas, Director of Graduate Studies. As such, each of my three data chapters contains its own Introduction and Discussion sections. The purpose of this General Introduction is therefore to provide a brief overview of previous work in the field, to outline the overall themes investigated in this thesis, and to describe how its three data chapters are related to one another.

Brief overview

Despite not knowing the principles of genetics, Darwin understood that heritable variation was essential for evolution (DARWIN 1859). Ultimately, genetic variation is generated by mutation. However, it is quite difficult to measure the properties of mutations because: 1) they are infrequent, 2) many only have very small phenotypic effects, and 3) natural selection tends to bias the mutations that are observed by biologists. One of the most important methods of studying mutations, the mutation accumulation (MA) experiment (BATEMAN 1959; MUKAI 1964; HALLIGAN AND KEIGHTLEY 2009), circumvents the latter problem by propagating very small populations. This decreases the efficacy of natural selection relative to genetic drift, allowing mutations to accumulate in a nearly unbiased manner.

However, mutations do not act in isolation. Interactions between mutations (called "epistasis") are pervasive (PHILLIPS 2008; DE VISSER *et al.* 2011). For example, if two genes have largely overlapping functions then the loss of either gene may have little effect on fitness, but loss of both genes may be lethal. Although epistasis is remarkably common, there is little evidence that interactions between random mutations are consistently biased to be positive (greater fitness than expected) or negative (lower fitness than expected) (ELENA AND LENSKI 1997; SANJUÁN *et al.* 2004; MAISNIER-PATIN *et al.* 2005; DE VISSER AND ELENA 2007; DICKINSON 2008;

HEILBRON *et al.* 2014).

In contrast to random mutations, epistasis between beneficial mutations appears to be more predictable. Several studies have shown that the fitness gain caused by a beneficial mutation tends to be attenuated in higher-fitness genetic backgrounds, a phenomenon called "diminishing returns epistasis" (CHOU *et al.* 2011; KHAN *et al.* 2011; KRYAZHIMSKIY *et al.* 2014; COUCE AND TENAILLON 2015). Therefore the "rules" that govern epistasis may be rather different for beneficial or random mutations.

Epistasis between a pair of mutations may itself be affected by mutations at other positions in the genome (called "higher-order epistasis") (WEINREICH *et al.* 2013). At the largest scale, the fitness associated with each possible genotype can be used to describe the "adaptive landscape" (WRIGHT 1932). Because it is difficult for humans to think in more than three dimensions, adaptive landscapes are most commonly depicted as a two-dimensional genotypic space in which distance denotes genetic similarity, and the fitness value of each genotype is projected onto the landscape in a third dimension (Figure 1). In reality, however, adaptive landscapes are highly multidimensional and as a result experiments have only been able to probe extremely small fractions of them.

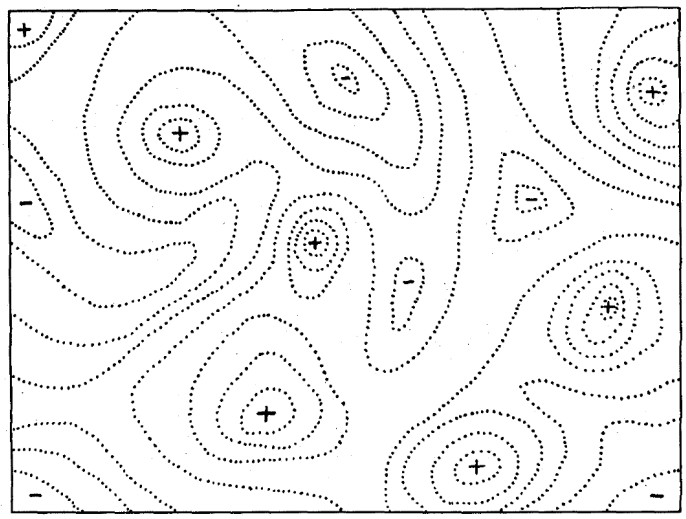


Figure 1 Sewall Wright's original depiction of an adaptive landscape (WRIGHT 1932). Contour lines represent genotypes of equal fitness the same way the contour lines on a topographic map represent positions of equal altitude.

One key property of adaptive landscapes that evolutionary biologists are particularly interested in is the extent to which they are "smooth" or "rugged". In a perfectly smooth landscape, all beneficial mutations confer increased fitness regardless of the genetic background into which they are inserted. Ruggedness is introduced into an adaptive landscape through sign epistasis—interactions between mutations such that the sign of the fitness effect (*i.e.* positive or negative) depends upon the genetic background. Ruggedness constrains the number of possible routes that adaptation can take (WEINREICH *et al.* 2006), and can lead to landscapes with multiple peaks (DE VISSER *et al.* 2009). In multi-peaked landscapes, populations can become trapped on lesser peaks, unable to attain higher fitness without first acquiring at least one deleterious mutation.

One of the main goals of my thesis is to explore various aspects of the fitness landscape of hypermutator *P. aeruginosa*. *P. aeruginosa* is a Gram-negative bacillus from the class Gammaproteobacteria. It is able to occupy a broad range of ecological niches, including soil and water, or as an opportunistic pathogen of plants, invertebrates, and vertebrates (RAHME *et al.* 1995). Unfortunately, this versatility allows *P. aeruginosa* to persist in hospitals where it poses a large threat as a nosocomial pathogen.

In particular, *P. aeruginosa* can chronically infect the airways of patients with cystic fibrosis—an autosomal recessive disorder that, amongst other things, leads to increased viscosity of lung mucus and reduced mucus clearance. In fact, *P. aeruginosa* lung infections cause more than 95% of death due to respiratory failure in cystic fibrosis patients (GOVAN *et al.* 2007). Hypermutator strains of *P. aeruginosa* commonly arise during chronic lung infections, exhibiting increased mutation rate and antibiotic resistance (OLIVER *et al.* 2000), and are associated with decreased patient lung function (OLIVER 2010). In this thesis I use normomutator (wild-type mutation rate) and Δ*mutS* hypermutator strains of *P. aeruginosa* PAO1 as model organisms to study various evolutionary phenomena.

Contents of the data chapters

In Chapter 1, I combined an MA experiment with whole genome re-sequencing to study the rate of spontaneous mutations, their distribution of fitness effects, and to test for epistasis between spontaneous mutations.

In Chapter 2, I performed a 70-generation selection experiment on 4 mutationally-degraded MA line endpoint clones from Chapter 1, re-adapting them to standard laboratory batch culture conditions. I used whole genome re-sequencing to investigate the molecular basis of adaptation and to probe the ruggedness of the adaptive landscape. Specifically, I was interested in testing whether the large number of mutations that separated each MA line endpoint would cause populations founded from different MA lines to take different evolutionary trajectories.

In Chapter 3, I measured the individual and combined effects of pairs of mutations—one beneficial and one deleterious—with particular focus on how the effect of a mutation changed depending on the fitness of the genetic background into which it was inserted.

Themes investigated in this thesis

My research has centred around two main themes. First, I have explored the relationship between epistasis and fitness, and how this relationship depends on whether the mutations involved are beneficial, deleterious, or random. In Chapter 1, I looked for unidimensional epistasis between random mutations as fitness decayed during MA. In Chapter 2, I investigated whether the amount of adaptation (beneficial mutations) observed during a selection experiment was related to initial fitness. In Chapter 3, I directly measured how the fitness effect of beneficial and deleterious mutations depended on the fitness of their genetic background by evolving all possible pairwise combinations of 3 beneficial and 3 deleterious mutations.

Second, much of the work in this thesis is centred on probing the adaptive landscape

of my study system. In Chapter 2, I tested whether populations that initially differed by a large number of mutations ended up climbing different fitness peaks by comparing their fitness gains and the mutations they acquired. In Chapter 3, I probed a small portion of a fitness landscape by measuring the fitness effect of a given mutation in several genetic backgrounds. In Chapter 1, I used an MA experiment to test for curvature in the shape of the landscape with distance from the optimum.

Chapter 1: Fitness is strongly influenced by rare mutations of large effect in a microbial mutation accumulation experiment

Karl Heilbron, Macarena Toll-Riera, Mila Kojadinovic, and R. Craig MacLean

Department of Zoology, University of Oxford, Oxford OX1 3PS, United Kingdom

Abstract

Our understanding of the evolutionary consequences of mutation relies heavily on estimates of the rate and fitness effect of spontaneous mutations generated by mutation accumulation (MA) experiments. We performed a classic MA experiment in which frequent sampling of MA lines was combined with whole genome resequencing to develop a high-resolution picture of the effect of spontaneous mutations in a hypermutator (ΔmutS) strain of the bacterium *Pseudomonas aeruginosa*. After ~644 generations of mutation accumulation, MA lines had accumulated an average of 118 mutations, and we found that average fitness across all lines decayed linearly over time. Detailed analyses of the dynamics of fitness change in individual lines revealed that a large fraction of the total decay in fitness (42.3%) was attributable to the fixation of rare, highly deleterious mutations (comprising only 0.5% of fixed mutations). Furthermore, we found that at least 0.64% of mutations were beneficial and probably fixed due to positive selection. The majority of mutations that fixed (82.4%) were base substitutions and we failed to find any signatures of selection on nonsynonymous or intergenic mutations. Short indels made up a much smaller fraction of the mutations that were fixed (17.4%), but we found evidence of strong selection against indels that caused frameshift mutations in coding regions. These results help to quantify the amount of natural selection present in microbial MA experiments and demonstrate that changes in fitness are strongly

influenced by rare mutations of large effect.

Introduction

Mutations are the ultimate source of genetic variation that natural selection acts upon. Understanding the rate at which mutations arise and the distribution of fitness effects of spontaneous mutations is therefore of central importance to the study of evolutionary biology (HALDANE 1937; KONDRASHOV 1988; PARTRIDGE AND BARTON 1993; CHARLESWORTH AND HUGHES 1996; CHARLESWORTH AND HUGHES 2000; HUGHES 2010; BANK *et al.* 2014). One of the most widely used methods for determining the rate and fitness effect of spontaneous mutations is the MA experiment. Following the pioneering work Bateman (BATEMAN 1959) and Mukai (MUKAI 1964), MA experiments involve propagating many replicate lines at very small effective population sizes so that the effect of natural selection is swamped out by that of genetic drift, allowing weakly selected mutations to accumulate randomly. The decline in mean fitness and increase in among-line variance in fitness are then used to indirectly infer mutation rate and effect estimates (BATEMAN 1959; MUKAI 1964; KEIGHTLEY 1994; GARCÍA-DORADO 1997; SHAW *et al.* 2002).

Recently, whole genome resequencing of MA lines has been used to directly measure the mutation rate in micro-organisms (LYNCH *et al.* 2008; LEE *et al.* 2012; NESS *et al.* 2012; SUNG *et al.* 2012a; SUNG *et al.* 2012b; LONG *et al.* 2013). In line with classic mutation rate estimates from reporter gene assays, the emerging consensus is that the genomic mutation rate is remarkably constant across DNA-based microbes, ~3×10^{-3} mutations/genome/generation (DRAKE 1991; LYNCH 2010). Accurate estimates of the fitness effects of spontaneous mutation, however, have remained elusive (EYRE-WALKER AND KEIGHTLEY 2007; HALLIGAN AND KEIGHTLEY 2009).

Because MA experiments rely on making comparisons among lines, they have traditionally focused on studying how fitness changes across as many lines as possible. An alternative approach is to combine whole genome resequencing in a smaller number of MA

lines of a hypermutator strain to allow a greater number of mutations to accumulate, thus increasing our ability to detect and quantify the amount of natural selection that occurs during microbial mutation accumulation experiments. Furthermore, whole genome resequencing directly determines the average number of mutations that accumulate between fitness measurements, allowing for improved estimates of the distribution of fitness effects of spontaneous mutations.

Natural selection must occur to some extent during microbial mutation accumulation experiments because colonies must grow big enough to become visible, resulting in an effective population size (N_e) >1. Beneficial and deleterious mutations should be subject to effective selection when $N_e s$ >1, where s is the absolute value of the fitness effect of the mutation, and the fluctuating population size of microbial MA experiments may further increase the efficacy of selection (OTTO AND WHITLOCK 1997). This may explain why many microbial MA experiments have reported results that are consistent with the fixation of some beneficial mutations as a result of positive selection (SHAW *et al.* 2000; JOSEPH AND HALL 2004; PERFEITO *et al.* 2007; DICKINSON 2008; TRINDADE *et al.* 2010; STEVENS AND SEBERT 2011). Studies have begun to combine both MA and whole genome resequencing in microorganisms (LYNCH *et al.* 2008; LEE *et al.* 2012; NESS *et al.* 2012; SUNG *et al.* 2012a; SUNG *et al.* 2012b; LONG *et al.* 2013), but none have detected a genomic signature of natural selection.

Using detailed fitness measurements and whole genome resequencing, we studied the evolutionary dynamics of eight replicate mutation accumulation lines of a hypermutator strain of the pathogenic bacterium *Pseudomonas aeruginosa*. MA lines were passaged through 28 single-cell bottlenecks followed by rapid population growth over a period of ~644 generations. Under this regime, we estimate that the effective population size of MA lines had a lower limit of ~16, which should be sufficient to prevent natural selection on the vast majority of spontaneous mutations. We determined the evolutionary dynamics of our lines with a high degree of precision by (1) directly measuring competitive fitness instead of a component of

fitness such as growth rate, and (2) measuring fitness at every second bottleneck to capture a small number of mutations between each time point. In line with recent work, we used deep whole genome sequencing to determine the genetic consequences of population bottlenecking, infer the molecular basis of altered fitness, and test for genomic signatures of natural selection during the MA procedure.

Consistent with previous MA experiments, we found that mean fitness decayed linearly over time. Detailed trajectories of fitness in individual lines coupled to whole genome sequencing revealed that rare, strongly deleterious mutations account for nearly half of the total loss of fitness. Furthermore, we found that positive selection resulted in the fixation of beneficial mutations, and that purifying selection was able to remove the majority of frameshift mutations.

Materials and Methods

Strains

The eight replicate clones used in this study were founded from the *P. aeruginosa* hypermutator strain PAO1ΔmutS, which was created by replacing *mutS*—part of the methyl-directed mismatch repair pathway—with the antibiotic resistance marker *aac1* using the Cre-lox system for gene deletion and antibiotic resistance marker recycling following the methods of Mandsberg *et al.* (MANDSBERG *et al.* 2011). Deleting *mutS* increases the mutation rate by ~70-fold in *P. aeruginosa* (TORRES-BARCELO *et al.* 2013), primarily by increasing the rate of transitions (MILLER 1996). The reference strain used to assess competitive fitness was PAO1-GFP. This strain was generated by integrating a constitutively expressed GFP marker at the chromosomal tn7 insertion site in *P. aeruginosa* PAO1 using the methods of Choi and Schweizer (CHOI AND SCHWEIZER 2006).

Mutation accumulation

Eight replicate mutation accumulation lines were generated by streaking randomly selected colonies of PAO1ΔmutS onto individual M9KB agar plates (glycerol, 10 g/liter; peptone, 20 g/liter; M9 salts, 10.5 g/liter; agar, 12 g/liter; and $MgSO_4$, 2 mL/liter). Plates were incubated at 37°C for 18 hr before repeating the process of picking a random colony and streaking it on a fresh plate. This process was repeated daily for 30 days. Each day, colonies would form from a single cell, which had doubled ~23 times, resulting in an N_e of ~16. Every second day, a portion of the randomly selected colony was suspended in a 50% v/v solution of glycerol and frozen at -80°C to be stored for competition assays. To ensure random selection of colonies, the last colony of the streak, which was not touching another colony, was selected. It is unlikely that random colony selection suffered a detection bias due to missing extremely small colonies; we sampled 14 regions between the visible colonies of our streaked plates and restreaked them, but did not detect a single instance of colony growth after 10 days.

Competitive fitness assay

Fitness of each line at each time point was determined relative to the PAO1-GFP strain. Strains were precultured in M9KB medium from frozen 50% glycerol stocks. Overnight cultures of each strain were mixed in M9KB broth at a ratio of ~80% mutant to 20% PAO1-GFP. The exact initial proportions were confirmed via flow cytometry. Mixtures were competed for 18 hr at 37°, with agitation at 200 rpm, and the final proportion was again measured by flow cytometry. We define the relative fitness of the mutant as the number of doublings that the mutant strain undergoes during the 18-hr competition divided by the number of doublings of the wild-type strain, given by the formula:

$$w_{\text{mutant}} = \frac{\log_2\left(\frac{N_{\text{final, mutant}}}{N_{\text{initial, mutant}}}\right)}{\log_2\left(\frac{N_{\text{final, wild type}}}{N_{\text{initial, wild type}}}\right)}$$

where w_{mutant} is the fitness of the mutant relative to the wild-type and N_{ij} is the number of either the mutant or the wild-type cells at either the beginning or the end of the competition. Each competition assay was performed in two experimental blocks with three replicate competitions per block. In some mutation accumulation lines, fitness became too low to accurately measure (final mutant proportion <10%) and thus these data have been excluded from all analyses except those pertaining to Figure 2 and the decay in average fitness over time. The inclusion of these inaccurate points does not change the statistical significance of any of the results presented.

Flow cytometry

Flow cytometry was used to determine the relative proportions of mutant and wild-type strains at the beginning and end of the competitive fitness assays. Bacterial cultures, diluted 200-fold in sterile filtered M9 salts, were prepared using deionized water to minimize background signal in the flow cytometer. Diluted mixtures were run on an Accuri C6 Flow Cytometer Instrument (BD Accuri, San Jose, CA) until 10,000 cells had been assayed. Events with a forward scatter value <10,000 or a side scatter value <8000 were excluded to prevent the false detection of small particles in the medium and electrical noise. To discriminate between GFP-tagged and untagged cells, cells were excited at a wavelength of 488 nm and fluorescence emissions between 518 and 548 nm were measured. There was a small overlap in the fluorescence profiles of tagged and untagged cells (*i.e.*, the most fluorescent untagged cells were slightly more fluorescent than the least fluorescent GFP-tagged cells), so pure cultures of PAO1 and PAO1-GFP were used as controls to correct for such spillover.

Whole genome sequencing

Illumina whole genome sequencing was performed on the first and last time point of each line, as well as on the five pairs of adjacent time points that showed the largest decrease in fitness. Raw sequencing data were analyzed using an in-house pipeline. Briefly, raw reads were filtered using the NGS QC Toolkit (PATEL AND JAIN 2012) and aligned against the reference genome using BWA (LI AND DURBIN 2009). Two approaches were used to call variants, GATK's Unified Genotyper (DEPRISTO *et al.* 2011) and SAMtools's Mpileup (LI *et al.* 2009). Identified variants were annotated with SnpEff (CINGOLANI *et al.* 2012). To detect structural variants, we combined two algorithms, Breakdancer (CHEN *et al.* 2009) and Pindel (YE *et al.* 2009). Finally, copy number variants (CNVs) were detected using Control-FREEC (BOEVA *et al.* 2012).

All differences between the *P. aeruginosa* PAO1 reference genome and the first time point of each bacterial line were excluded, leaving only mutations that accumulated throughout the experiment. Sequences from intermediate time points were treated as sequences from end points. All mutations found in intermediate time points were found at the end points except for one that fell in a mutation hotspot.

Testing for selection on base substitutions

To test for selection on base substitutions in protein coding genes, we estimated the expected number of protein altering mutations, under the assumption that synonymous mutations are effectively neutral. Specifically, since almost all base substitutions in our experiment were transitions (99.5%), we calculated the neutral mutation rate of each of the four bases to its partner (A→G, G→A, C→T, and T→C) using the observed synonymous mutations in our experiment. Given these mutation rates, we used the nucleotide composition and codon usage of *P. aeruginosa* proteins to estimate the rates of nonsynonymous and synonymous mutations (d_N/d_S ratio), as well as the rates of stop-gain, stop-loss, and intergenic mutations. To test for a deviation from the neutral expectation, we tested the null hypothesis

that the proportion of mutations in a given class (nonsynonymous, truncation, or intergenic) relative to the number of observed synonymous mutations is equal to the predicted ratio calculated using the synonymous mutation rate. This hypothesis was tested using the normal approximation of the binomial distribution (ZAR 2010).

Repetitive regions

The RepeatMasker program (SMIT *et al.* 1996-2010) was used to screen the PAO1 genome for simple repeats, interspersed repeats, and low-complexity DNA sequences. Homopolymeric tracts of single nucleotide repeats ranging from 4 to 20 bases were identified using the dreg program, implemented in the EMBOSS package (RICE *et al.* 2000).

Magnitude of selection against indels in coding regions

The percentage of indels in repetitive coding regions removed by natural selection was calculated under the assumption that indels in the repetitive noncoding genome are neutral. The expected number of indels in repetitive coding regions before natural selection was calculated by dividing the observed number of "neutral" mutations in repetitive noncoding regions by the fraction of repetitive elements that are in noncoding regions (21.8%) and multiplied this value by the fraction of repetitive elements that are in coding regions (78.2%). The percentage of indels removed due to natural selection is then 1 - observed/expected. If mutations in noncoding repetitive regions are not neutral, then this method will generate a lower limit estimate.

Core genes

Precomputed pairwise reciprocal best BLAST hits for 36 *Pseudomonas* species were downloaded from the *Pseudomonas* Genome Database (WINSOR *et al.* 2011). The core genome for *P. aeruginosa* PAO1 was defined as the set of PAO1 genes that had pairwise reciprocal best

BLAST hits in the 35 remaining *Pseudomonas* species. We found a total of 1435 core genes.

Clusters of Orthologous Groups analysis

A list of *P. aeruginosa* PAO1 genes with annotated Clusters of Orthologous Groups (COGs) categories (TATUSOV *et al.* 2000) was downloaded from the National Center for Biotechnology Information. This list was intersected with the list of genes that had experienced at least one mutation during our experiment. Genes with annotated mutations and COG categories were compared to the rest of the genes in the PAO1 genome that were unmutated, but had been assigned a COG category. *P*-values were computed using Fisher's exact test and corrected for multiple testing using the false discovery rate method (BENJAMINI AND HOCHBERG 1995).

Statistical analysis and simulations

All statistical analyses were conducted in R (version 2.15.0) (R DEVELOPMENT CORE TEAM 2012). All statistical tests are reported as a *P*-value and the value for the test statistic with a subscript indicating the degrees of freedom. All tests use $\alpha = 0.05$ and, where applicable, are two tailed.

Simulations were used to generate the expected distribution of the number of mutations per gene, given the substantial variation in gene length in the *P. aeruginosa* genome (mean: 830 bp, 95% confidence interval: 247–2786 bp). The lengths of all genes in the *P. aeruginosa* genome were obtained from the *Pseudomonas* Genome Database (WINSOR *et al.* 2011). In each simulation, mutations (either synonymous or nonsynonymous) were randomly distributed across a simulated genome, using the same number of mutations as was detected in our experiment. The number of mutations per gene was recorded and results were averaged across 100 simulations.

Results

Here we present the results from a ~644-generation-long mutation accumulation experiment in eight replicate MA lines. We measured the fitness of each MA line every 2 days, providing a high-resolution picture of the evolutionary dynamics of heavily bottlenecked bacterial populations. We performed whole genome resequencing on multiple time points of each line to determine the molecular nature of mutations fixed under conditions of relaxed natural selection.

Whole genome resequencing identified 944 mutations in the eight mutation accumulation lines. Sanger sequencing of a random sample of these mutations confirmed 35/35 mutations (Supporting Information, Table S1), indicating a very low false positive rate. As expected, mutations were Poisson distributed across MA lines (one-sample Kolmogorov–Smirnoff test: $P = 0.521$, $D = 0.270$) with an average of 118 mutations fixed per line and an average of 8.4 mutations fixed between each adjacent time point. This equates to a per base pair mutation rate of 2.95 (± 0.21 SE) x 10^{-8} mutations/site/generation and a genomic mutation rate of 0.18 (± 0.01 SE) mutations/genome/generation. Given that the hypermutator strain used in this study increases the mutation rate by ~70-fold (TORRES-BARCELO *et al.* 2013), this estimated genomic mutation rate is in line with the consensus bacterial genomic mutation rate of ~3 x 10^{-3} mutations/genome/generation (DRAKE 1991; LYNCH 2010).

Of the 944 mutations, 778 (82.4%) were base substitutions, 164 (17.4%) were short indels (<10 bp), and 2 (0.2%) were large structural variations, consisting of a partial gene duplication event (*pvdD*) and a 1880-bp intergenic deletion. Insertions were ~2.5-fold more common than deletions (118 insertions *vs.* 46 deletions) (Figure 1). As is typical for a ΔmutS hypermutator strain, almost all base substitutions were transitions (774/778 = 99.5%), and G:C→A:T transitions (478) were ~60% more common than A:T→G:C transitions (298).

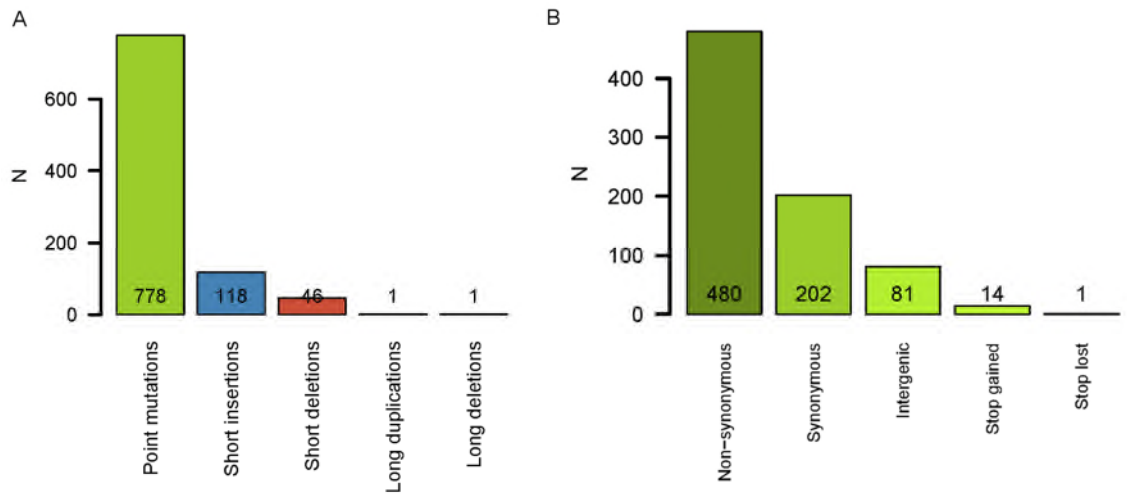


Figure 1 Types of mutations accumulated. (A) The distribution of accumulated mutations according to type of mutation. Indels <10 base pairs long were considered to be "short." (B) Further information on the effects of point mutations.

As expected, the average fitness of the hypermutator populations decreased significantly over time (Figure 2; ANOVA: $P = 1.68 \times 10^{-6}$, $F_{1,13} = 67.409$), indicating that the average effect of spontaneous mutations was deleterious and that recurrent population bottlenecks inhibited the action of natural selection (mean mutational fitness effect = -0.16%). Adding a quadratic term did not significantly improve the fit of a model with the logarithm of fitness as the dependent variable and transfer number as the independent variable (partial F-test: $F_1 = 2.606$, $P = 0.132$), indicating that there was no detectable unidimensional epistasis. In some lines, fitness became so low that it was no longer possible to reliably measure (Figure 3). These data are included in Figure 2 to prevent bias, but excluded from subsequent analyses. The average fitness of bottlenecked nonhypermutator control lines did not change significantly over the course of the experiment (ANOVA: $P = 0.712$, $F_{1,118} = 0.137$), indicating that the loss of fitness in hypermutator lines was due to mutation accumulation.

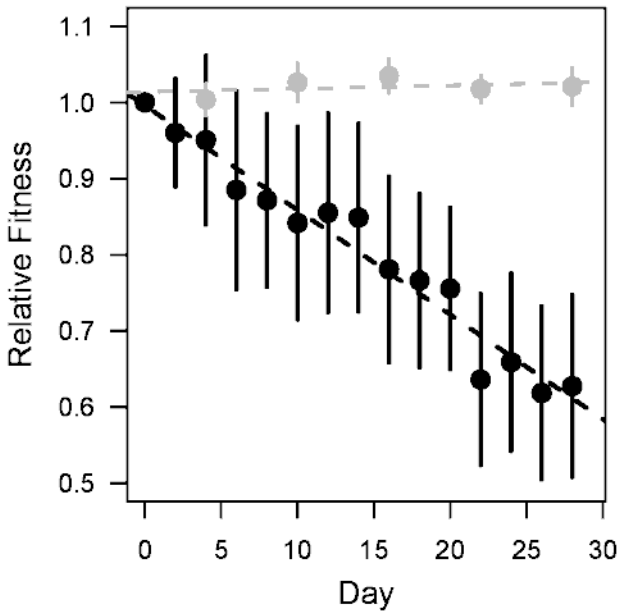


Figure 2 Average fitness decays in mutation accumulation lines. Plotted points show the mean fitness (± SE) of hypermutator lines (solid symbols, n = 8) and control lines (shaded symbols, n = 4) that were passaged through 28 daily bottlenecks, which correspond to ~644 generations of mutation accumulation. The fitness of hypermutator lines rapidly declined, but the fitness of control lines did not change over the course of the experiment (ANOVA: $F_{1,3} = 0.436$, $P = 0.556$). Note that in some MA lines, fitness decayed to the point where it was not possible to measure fitness reliably, but these data are included to prevent bias.

Fitness data

Unlike the linear decrease observed for average fitness, the evolutionary trajectory of individual lines was much more complex (Figure 3). The net change in the fitness of MA lines ranged from -1 to -27% (mean: -13% ± 9 SD). A large portion of the net decrease in fitness of each line was due to a single drop between adjacent time points (we hereafter refer to a pair of adjacent time points as a "step"). Specifically, on average, 42.2% (± 12.9% SD) of the total decrease in fitness between the first and last time point in an individual MA line (excluding any beneficial steps) was due to the largest deleterious step in that line. Furthermore, the four most deleterious steps across all lines accounted for 42.3% of the total fitness decrease throughout the entire experiment.

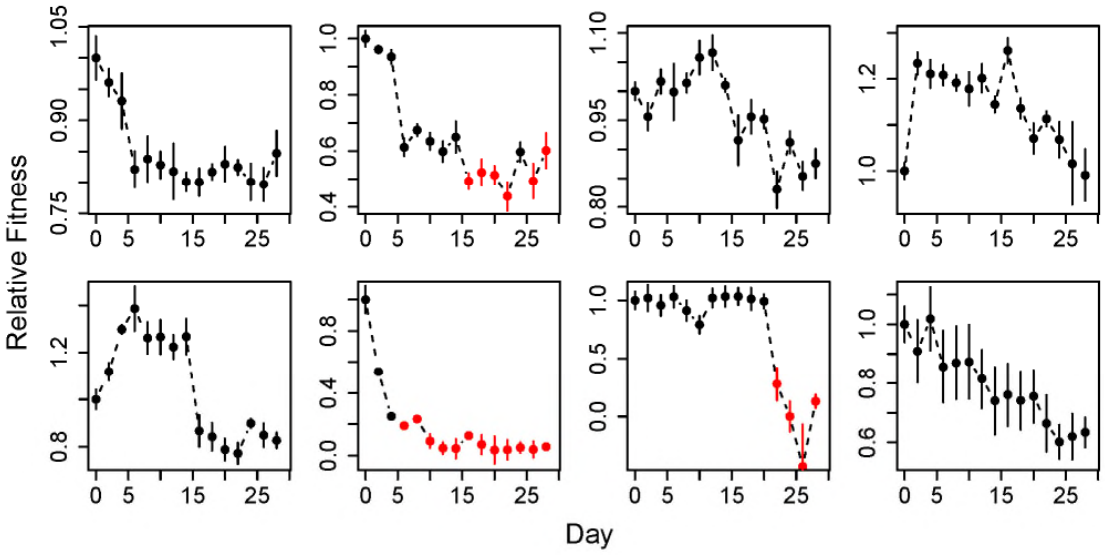


Figure 3 Fitness trajectories for individual mutation accumulation lines. The mean (± SE; n = 6) fitness of individual hypermutator lines through time. Red data points indicate that fitness is too low to measure accurately. The mean fitness (± SE; n = 6) of individual hypermutator lines through time. Red data points indicate that fitness is too low to measure accurately. The y-axis of each plot is scaled differently to maximize the resolution of evolutionary dynamics within a single line.

To determine whether these large drops in fitness were caused by (1) the accumulation of a greater number of mutations than other steps or (2) the accumulation of mutations of larger effect, we performed whole genome sequencing on the four largest deleterious steps across all MA lines, as well as on an exceptionally large deleterious step, which caused the fitness of its MA line to drop to an undetectable level. These steps did not contain a significantly greater number of mutations than the remaining steps (mean of five largest steps: 9.0 mutations, mean of remainder: 7.9 mutations, paired *t*-test: $P = 0.285$, $t_4 = 1.235$). However, these large deleterious steps showed a significantly higher frequency of mutations in highly conserved core genes than other steps (χ^2 goodness-of-fit test: $P = 0.049$, $\chi^2_1 = 3.882$; Table S2). Therefore, large drops in fitness are due to mutations in more important genes rather than due to a greater number of mutations.

Although the average fitness effect of a step was deleterious, there were numerous steps in which fitness increased (Figure 4). To confirm the presence of steps containing beneficial mutations, we repeated the competitive fitness assays for the 11 steps with the

largest increases in fitness. Even after false discovery rate correction (BENJAMINI AND HOCHBERG 1995), fitness increased significantly ($P < 0.05$) in 6/89 (6.7%) of the measurable steps. Because steps where fitness increased were rare, it is likely that each of these steps only contained a single beneficial mutation. This implies that at least six beneficial mutations were fixed during the mutation accumulation experiment, which corresponds to 0.64% of all mutations that were fixed during the experiment.

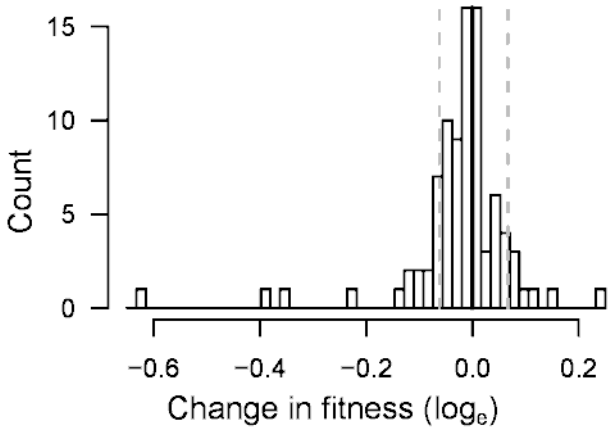


Figure 4 Changes in fitness for individual "steps". The distribution of fitness changes for each step in the mutation accumulation experiment across all eight hypermutator lines. Each step represents the difference in fitness between successive assays for an MA line (~8.4 mutations accumulated/step). The solid line depicts no change in fitness and the area between the dashed shaded lines is the area in which $N_e s < 1$, where N_e is the harmonic mean of population size over time (although this may be an underestimate) (OTTO AND WHITLOCK 1997).

Signatures of natural selection

Selection on base substitutions in protein coding genes: The vast majority of protein-altering base substitutions were nonsynonymous mutations, but the ratio of the rate of nonsynonymous mutations to silent mutations ($d_N/d_S = 1.08$) did not differ significantly from the neutral expectation of 1 (Table 1; *Z*-test: $Z = 0.92$, $P = 0.26$). We observed only a single loss-of-stop mutation, but this was similar to our predicted number of 1.4. Truncation mutations that introduce a premature stop codon were much more frequent (n = 14), but this was not

significantly different from the neutral expectation of nine truncation mutations (*Z*-test: $Z = 1.63$, $P = 0.10$).

Table 1. Testing for selection on single base pair substitutions

Protein effect	Observed	Expected
Non-synonymous	480	444.38
Intergenic	80	84.33
Stop-gain	14	8.94
Stop-loss	1	1.41

The number of observed single base pair substitutions relative to the neutral expectation, as determined from the synonymous mutation rate and genome composition of *P. aeruginosa*. The observed number of mutations does not differ from the neutral expectation for any functional category of mutation.

Selection on coding and noncoding regions: Protein coding sequence accounts for 89.4% of the *P. aeruginosa* genome and so we expected that if no natural selection has occurred during the MA experiment then ~89.4% of mutations will have occurred in protein coding sequences. We found that the percentage of mutations (short indels and base substitutions) that occurred in coding regions was 85.4% (804/942), which was significantly different from the neutral expectation of 89.4% (χ^2 goodness-of-fit test: $P < 0.001$, $\chi^2_1 = 15.888$). Interesting patterns arose when we analyzed base substitutions and short indels separately.

We found that the percentage of base substitutions in coding regions (89.6%, 697/778) and intergenic regions (10.41%, 81/778) was not significantly different from the neutral expectation (χ^2 goodness-of-fit test: $P = 0.833$, $\chi^2_1 = 0.045$). This result may be confounded because intergenic regions contain a larger proportion of repetitive DNA than coding regions (intergenic: 7.2%, coding: 3.1%), but when we restricted our analysis to repetitive regions we still observed that the percentage of base substitutions that fell in coding (4.3%) and intergenic

(16.5%) repetitive regions did not differ from the neutral expectation (χ^2_1 goodness-of-fit test: P = 0.181, χ^2_1 = 1.791).

Selection on indels: In contrast to base substitutions, we found significantly fewer indels in coding regions than expected (observed: 107/164 = 65.2%; expected: 89.4%; χ^2 goodness-of-fit test: $P < 0.0001$, χ^2_1 = 100.236). Again, this difference could be confounded because intergenic regions contain a larger proportion of indel-prone repetitive DNA, but we also found significantly fewer indels in repetitive coding regions (observed: 103/160 = 64.4%; expected: 78.2%; χ^2_1 goodness-of-fit test: $P < 0.0001$, χ^2_1 = 17.920) than expected in the absence of selection. This indicates strong purifying selection against frameshift mutations. In fact, these data suggest that at least 49.6% of frameshift mutations are sufficiently deleterious to be removed by natural selection, even under a regime of intense bottlenecking. Despite selection against frameshift mutations, we still found 106 frameshifts in our experiment. Almost all of them (101/106 = 95.3%) overlapped with homopolymeric tracts of C (ranging from 4C to 8C) or G (ranging from 5G to 8G). There were significantly more frameshifts located near the N terminus of the protein than expected, given the distribution of homopolymeric tracts in the *P. aeruginosa* genes (Figure 5; one-sided exact binomial test: $P = 0.037$). We found no significant difference for frameshifts near the middle (one-sided exact binomial test: $P = 0.453$) or near the C terminus of the protein (one-sided exact binomial test: $P = 0.063$).

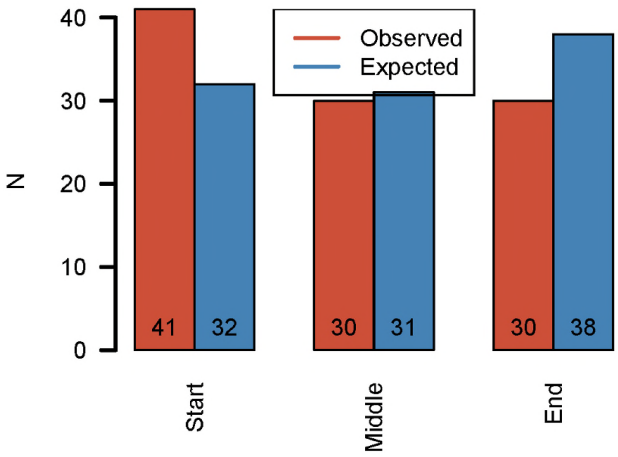


Figure 5 The distribution of indel mutations in proteins. Comparison between the observed and expected position of frameshifts in coding regions. Proteins were divided into three equal pieces and we counted the number of frameshifts (overlapping with homopolymeric tracts) that fell in each section. Expected frequencies were computed by counting the number of homopolymeric tracts in the *P. aeruginosa* PAO1 proteome that fall in each section. The differences between observed and expected values were statistically significant for the N-terminal third of proteins (one-sided exact binomial test: $P = 0.037$).

Tests for parallel evolution: Previous work has shown that exposing replicate microbial populations to a similar selective pressure results in parallel adaptation at a molecular level in both lab experiments (WICHMAN *et al.* 2000; SEGRÈ *et al.* 2006; BARRICK *et al.* 2009) and clinical populations (HUSE *et al.* 2010; LIEBERMAN *et al.* 2011). To test for parallel evolution at the level of individual genes, we compared the distribution of the number of mutations fixed per gene in the eight MA lines, with the distribution expected based on the lengths of the genes in the *P. aeruginosa* genome (Figure S1; see Materials and Methods for details on calculating the expected distribution). We found no deviation from the expected distribution for synonymous mutations (χ^2 goodness-of-fit test: $P = 0.643$, $\chi^2_2 = 0.883$). On the other hand, we found significantly fewer parallel nonsynonymous mutations than expected (χ^2 goodness-of-fit test: $P < 0.0001$, $\chi^2_2 = 19.302$), which does not support the hypothesis that natural selection was capable of causing parallel evolution on the genomic scale in these MA lines. Rather, longer genes simply had more mutations than smaller genes (Figure S2): genes with one or more mutations were significantly longer than genes without mutations (Kolmogorov–Smirnov test,

$P < 0.001$).

It is also possible that parallel evolution could act on levels higher than the gene. We analyzed our mutation data for evidence of over- or underenrichment of mutations in COGs—genes that share a common function. After false discovery rate correction (BENJAMINI AND HOCHBERG 1995), we found a significant underrepresentation of mutated genes involved in transcription (Table S3; Fisher's exact test: $P = 0.023$, Fisher's odds ratio$_1$ = 0.530), suggesting that mutations in these genes tend to have highly deleterious effects.

Core genes: We observed that large drops in fitness during the MA experiment were associated with the accumulation of mutations in core genes (Figure 2), and so we sought to determine whether natural selection was effective against mutations in these genes. Surprisingly, there was no significant underrepresentation of mutations in core genes (Fisher's exact test: $P = 0.611$, Fisher's odds ratio$_1$ = 1.051) despite their potentially large deleterious effects on fitness.

Discussion

Mutations are rare events that often lead to small changes in fitness, and these properties of mutations make it intrinsically difficult to directly study the evolutionary consequences of mutation. Our experiment, which combined a classic mutation accumulation experiment with powerful whole genome resequencing technology, found that 42.3% of the decrease in fitness in our lines was driven by 4.5% of the steps with highly deleterious effects on fitness. Given the rarity of large drops in fitness, the most parsimonious explanation is that each one of these drops was driven by a single highly deleterious mutation. Under this assumption, the 42.3% of the decrease in fitness in our experiment was driven by 0.5% of the mutations fixed, which is consistent with previous work in *Caenorhabditis elegans* (DAVIES *et al.* 1999). The mean mutational effect, $s = -1.6 \times 10^{-3}$, is similar to previous work in *Saccharomyces cerevisiae* ($s = -6 \times 10^{-3}$), in which whole genome resequencing and MA were combined (LYNCH

et al. 2008) and, as expected, is approximately one to two orders of magnitude smaller than previous microbial MA studies that did not use whole genome resequencing and were therefore unable to detect neutral mutations (HALLIGAN AND KEIGHTLEY 2009; TRINDADE *et al.* 2010). We also found evidence of both positive and negative selection in our MA experiment, demonstrating that the results of our experiment cannot be interpreted as a proxy for the effects of spontaneous mutation alone.

Beneficial mutations

Previous studies in *Arabidopsis thaliana* (SHAW *et al.* 2000), *Escherichia coli* (PERFEITO *et al.* 2007; TRINDADE *et al.* 2010), *Streptococcus pneumoniae* (STEVENS AND SEBERT 2011), and *S. cerevisiae* (JOSEPH AND HALL 2004; DICKINSON 2008) have also found evidence that beneficial mutations are fixed during mutation accumulation experiments. Our experimental approach allowed us to experimentally demonstrate that it is highly likely that at least 0.64% of the mutations that fixed during our MA experiment were beneficial. For these mutations to have been fixed by drift, the beneficial mutation rate in a nonhypermutator population with a genomic mutation rate of 3×10^{-3} mutations/genome/generation would have to have been ~5×10^{-6} mutations/genome/generation, which is two to three orders of magnitude higher than existing estimates (GERRISH AND LENSKI 1998; MIRALLES *et al.* 1999; IMHOF AND SCHLOTTERER 2001a; ROZEN *et al.* 2002; BARRETT *et al.* 2006) (but for an exception, see (PERFEITO *et al.* 2007)). Instead, we argue that positive selection was able to drive the fixation of beneficial mutations in our experiment. Consistent with this idea, five of the six significantly beneficial mutations that fixed were sufficiently beneficial that $N_e s$ was >1.

Tests for selection at a molecular level

In agreement with recent microbial mutation accumulation experiments that have used whole genome resequencing, we found no evidence of selection on base substitutions,

including nonsynonymous mutations (LYNCH *et al.* 2008; LEE *et al.* 2012; NESS *et al.* 2012; SUNG *et al.* 2012a; SUNG *et al.* 2012b; LONG *et al.* 2013). Additionally, we found no evidence of positive selection on the same genes in different MA lines. Surprisingly, we found that nonsynonymous mutations in highly conserved core genes can have strong deleterious effects on fitness (Figure 2), and yet we found no evidence that these mutations were removed by natural selection. The most striking evidence of selection at a genetic level comes from the lack of short indel mutations in coding regions. We estimate that negative selection prevented the fixation of at least 50% of indels in coding regions. In contrast, we did not find any evidence of an underrepresentation of base substitutions that generated a premature stop codon, implying that the absence of indels in coding regions is due to selection against frameshifts, and not selection against gene loss.

Despite strong selection, we still found that frameshifts comprise 13.2% of all mutations in coding regions. This high incidence of frameshifting could be because 95.3% of frameshifts overlapped with homopolymeric tracts. Homopolymeric tracts are hypermutable: they are highly prone to gaining or losing repeats through slippage, thereby producing indels. Consistent with recent work (ORSI *et al.* 2010; LIN AND KUSSELL 2012), we found a significant overrepresentation of frameshifts at the 59 end of genes and underrepresentation at the 39 end (given the distribution of homopolymeric tracts in the PAO1 genome). Although the reasons for the enrichment of 59 frameshifts is unclear, possible explanations include: (1) 59 frameshifts tend to create shorter proteins and thus may be less prone to forming toxic aggregations; (2) intergenic regions in *P. aeruginosa* are very short and 39 indels may knock out downstream genes; and/or (3) 59 indels are more likely to destroy gene function, which may be beneficial in some circumstances. For example, Moxon *et al.* (MOXON *et al.* 2006) have proposed that simple sequence repeats (such as homopolymeric tracts) are localized hypermutation targets and a mechanism for adaptation. Moreover, standing genetic variation in homopolymeric tracts has been shown to drive the adaptation of *Campylobacter jejuni* to a

novel host (JEROME *et al.* 2011).

Implications for mutation accumulation experiments

It is important to emphasize that our experiment differed from most previous MA experiments because we used a hypermutator strain. To what extent is this likely to have biased our results? Hypermutators produce an altered spectrum of spontaneous mutations (*e.g.*, bias toward transitions), which can have important evolutionary implications when strong selection acts on a small number of sites in the genome (COUCE *et al.* 2013) (*e.g.*, some cases of high-level antibiotic resistance). In our system, frameshifts experienced much stronger selection than any other class of mutation, and it is possible that using a ΔmutS hypermutator altered the rate of appearance of indel mutations relative to base substitutions (MARVIG *et al.* 2013). However, by using a hypermutator we were able to detect a sufficiently large number of mutations to analyze the effects of relatively rare types of mutation, such as indels, which have traditionally been overlooked in MA studies.

Conclusion

In conclusion, we find that fitness decays in recurrently bottlenecked populations of hypermutator *P. aeruginosa* because of the fixation of many weakly deleterious mutations and a few highly deleterious mutations. We argue that this pattern of punctuated decay of fitness arises for two reasons. First, most mutations carry little, if any, fitness cost in a laboratory environment, but a substantial fraction of mutations are highly deleterious. Our results suggest that weakly deleterious mutations tend to be intergenic and nonsynonymous mutations, while highly deleterious mutations tend to be indels and mutations in core genes. Second, we find that recurrent bottlenecking does not completely compromise the efficacy of natural selection in microbial mutation accumulation experiments, although large deleterious mutations are unlikely to play a substantial role in the evolution of natural populations. We hope that this

study will pave the way for future work aimed at understanding: (1) why frameshift mutations are subject to such strong selection, (2) how bacteria adapt to the deleterious effects of spontaneous mutations, and (3) how the molecular basis of spontaneous mutation is linked to the fitness effects of mutations in natural populations.

Acknowledgements

We thank Antonio Oliver for providing us with the PAO1ΔmutS strain. We thank the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics (funded by Wellcome Trust grant 090532/Z/09/Z and Medical Research Council hub grant G0900747 91070) for the generation of the sequencing data.

Supplementary Material

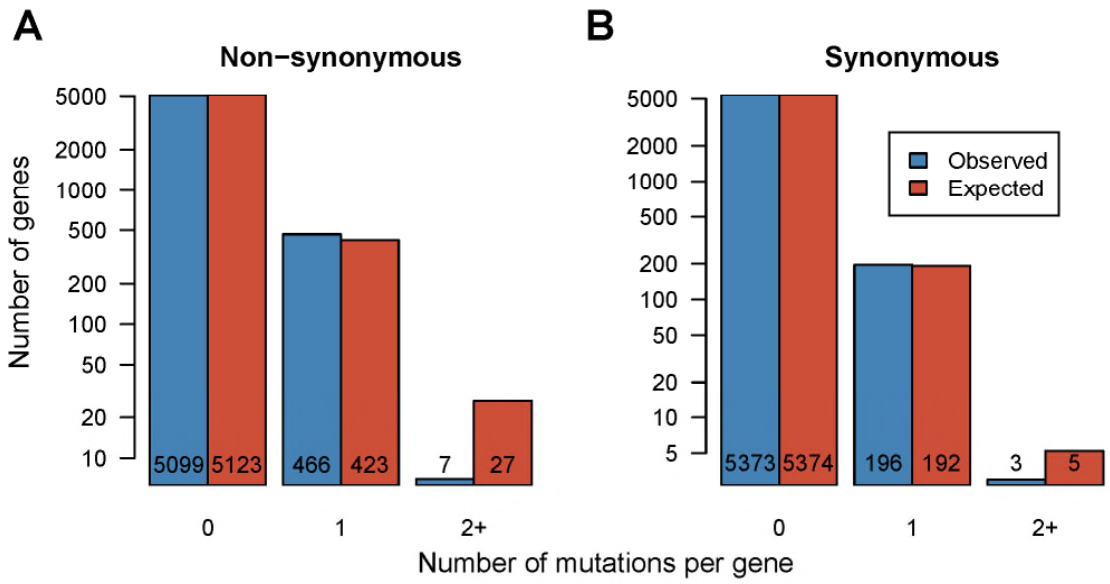


Figure S1 Testing for the presence of parallel evolution at the level of the gene. The observed and expected distributions of the number of mutations per gene are presented for (A) non-synonymous and (B) synonymous mutations.

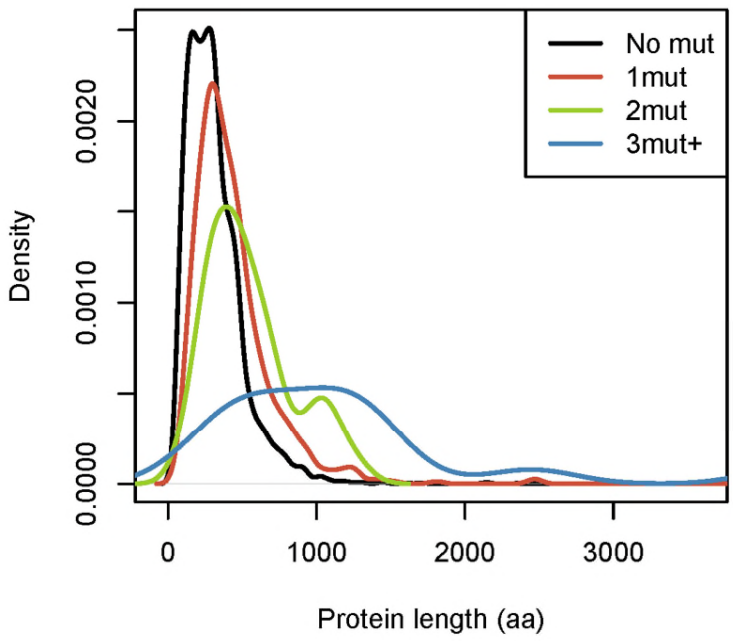


Figure S2 Genes that accumulated more mutations tended to be longer. Mutated genes were grouped according to the number of mutations that they experienced and their gene length distribution was compared to the distribution for non-mutated genes. Differences between distributions were computed using Kolmogorov-Smirnov tests; all pairwise comparisons were statistically significant (Kolmogorov-Smirnov test: $P<0.001$).

Supplemental Methods: whole genome re-sequencing

Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) with the following amendments to the protocol: cells were pelleted by centrifuging for 2 minutes at 13,000 RPM; following the protein precipitation step solutions were vortexed for 30 seconds, incubated on ice for 10 minutes, and centrifuged for 10 minutes at 13,000 RPM. Extracted DNA was rehydrated with 200-300µL of "Buffer EB" (Qiagen, San Francisco, USA - UK catalogue number: 19086) and DNA concentration was assessed using the QuantiFluor™ dsDNA System (Promega, Madison, WI, USA). Genomic DNA was then diluted in "Buffer EB" to 50 ng/µL. To ensure sample purity the diluted genomic DNA was run on a 0.7% agarose gel and a NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA) to ensure sample purity. All sequencing was conducted at the Welcome Trust Centre for Human Genetics using the HiSeq2000 instrument and 100bp paired-end reads (24 samples) and the MiSeq instrument and 150bp paired-end reads (2 samples).

Sequencing data was initially filtered using the NGS QC Toolkit (PATEL AND JAIN 2012), which discarded around 9% (± 0.392 SD) of the reads for each sample. Reads were trimmed from the 5' and/or 3' end if the PHRED quality score was below 20. After the trimming step, reads shorter than 50bp (Hiseq) or 75bp (Miseq) were eliminated. Reads were also discarded if more than 2% of their bases were ambiguous or if more than 20% of their bases had a PHRED quality score below 20.

Filtered reads were mapped to the *P. aeruginosa* PAO1 reference genome (NC_002516.2) using BWA (LI AND DURBIN 2009). The average median depth of coverage across lines was 232-fold (HiSeq) and 48-fold (MiSeq) with a median of 99.1% (HiSeq) and 98.9% (MiSeq) of the genome covered to a depth of at least 5 reads. Mapped reads were processed to increase the quality of the variant calling: 1) reads with multiple best hits were discarded; 2) duplicated reads were eliminated using the MarkDuplicates tool from the Picard package (http://picard.sourceforge.net); 3) reads were locally realigned around indels using

RealignerTargetCreator and IndelRealigner from the GATK package (DEPRISTO *et al.* 2011) because these regions are prone to misalignment; and 4) reads mate information was fixed and sorted using the FixMateInformation command in the Picard package (http://picard.sourceforge.net).

Variant calling was performed on the processed mapped reads using two different approaches: GATK's Unified Genotyper (DEPRISTO *et al.* 2011) and Samtools's mpileup (LI *et al.* 2009). VCFtools (vcf-annotate) (DANECEK *et al.* 2011) and GATK toolkit (VariantFiltration) (DEPRISTO *et al.* 2011) were used to filter the raw variants for strand bias, end distance bias, base quality bias, SNPs around gaps, low coverage and erroneously high coverage. High quality variants were subsequently annotated using SnpEff (CINGOLANI *et al.* 2012).

Structural variants were detected using three different approaches. First, Breakdancer (CHEN *et al.* 2009) was used to predict five types of structural variants—deletions, insertions, inversions, inter- and intra-chromosomal translocations—using information from read pair mapping. The output of Breakdancer was given to Pindel (YE *et al.* 2009), which can use calls from other programs to increase its performance. Pindel can infer deletions, short insertions, long insertions, inversions, tandem duplications, and breakpoints using a split-read approach. Finally, Control-FREEC (BOEVA *et al.* 2012) was used to detect copy number variants (CNVs). Control-FREEC identifies CNVs using depth of coverage and normalization by GC content. Regions with low mappability can be excluded from the analysis by providing Control-FREEC with mappability tracks. Mappability tracks were created using GEM library (gem- mappability) (MARCO-SOLA *et al.* 2012).

Table S1 Mutations verified by Sanger sequencing.

MA line	Genomic position	Type of mutation	Reference nucleotide	New nucleotide	Forward primer sequence	Reverse primer sequence
1	145116	SNP	C	T	CACCATGGGCGCACATTC	CGGAGAAGTCGAGGGTGC
1	1520010	SNP	C	T	AGGACGAAGCGAATCCCAG	GGCATCTGCATCGAAGACCT
1	1619035	SNP	G	A	CGGTGGACATGGGCGGCTAC	GATGGAAAGCGCCCAGGGCCA
1	3747910	Deletion:1	TGGGGG	TGGGG	TGGCCCATATCGGCGTGCTC	GCCACCATCCACCAGCAGCC
1	5681569	SNP	G	A	ACAAGATCTACCTGGGCAAC	ATCCCGCTCTTTTCCACCTG
2	882486	SNP	T	C	ACCATCGACCTCTACCTGCT	AGGTTTCGCTTGCGGGTAG
2	3547996	SNP	G	A	AAAAGCTGCCGAAGCCACGG	GCCGCGTCATCAGCCTCTCC
2	4388038	SNP	G	A	CCGGGCAGGAAGCCGATGTC	ACCATTCCCTCGCCGGCTCT
2	5145236	Insertion:1	GCCCCC	GCCCCCC	CTATAGACGTCCGCTCCCG	GTGCAGTTCCCGTTCGCC
2	5957160	SNP	C	T	GCAAGCAGGGCTACGAAGTC	GAAAACGGCGTGCACCTG
3	74465	Insertion:1	GCCCCCCCC	GCCCCCCCCC	TGGTGGTCGGCAGTTGCGAG	GCCCCATGCGTCCACTCACC
3	1470605	SNP	A	G	GACGCCGCTGGCGGTGTACTG	TGTGCGGCACGCCTTCGATT
3	4561412	SNP	A	G	GTGCCGTAGGGCCCCGGATA	GGTCCTGGGCGGCAACTACG
3	4780397	SNP	T	C	ACGGTGCAGCTGGGAGACGA	AGCTGGGGAACGCTGATGGC
4	788414	Insertion:1	TG	T	TCGCGACCGACATGCAACTGA	TTCACGGGGGCGATTACGAG
4	975675	SNP	C	T	AGGTGGCCGGTCGTCAAC	CTCATGTGCGCGGGTAG
4	4556274	SNP	T	C	CTGGCGCTGCGCTACCTGTT	GATAGCCGGCTGGCGCAACA
4	5158358	SNP	A	G	GTCCAGGCGGTGCCCGATAC	TCACGTCGCTGATGTTTTCC

MA line	Genomic position	Type of mutation	Reference nucleotide	New nucleotide	Forward primer sequence	Reverse primer sequence
4	5979956	SNP	G	A	CCGGCGCATGCACAACAAGC	CGCATCCCAGATCGCCTCGG
5	3464663	SNP	G	A	GATCCAGCAGTGCCCGAG	AGTTGTACCGGCGTTATCCC
5	3582674	SNP	G	A	TGCCGATACAGCGCTTGAG	TTTCACCGGTTTGCTGATGC
5	4012262	SNP	T	C	CGGGATTAGCGGCTCATTCA	TTCTCCGGGAGTGACCATGA
5	4063465	SNP	G	A	CAGGGACAATCGCTCGGATG	GCGTTACCGTCATTTTTCCCG
5	4409497	SNP	T	C	ACCCACTTCCTCCAGCCG	AGGGTGTTGAGCAGGTGC
5	4417437	SNP	G	A	GGAAGGACTCCGCCGCCAGT	GGTAGTCCTGAACAGCAGCGCC
5	5075369	SNP	G	A	CAAGCCCAGTTGCGCCACCA	TGCCGGCCATGCGTGACTAC
5	5246015	SNP	A	G	GGTGACCAGGATGTGCTTGA	GCTGGAACGGGAACTCTTCG
5	5432314	SNP	G	A	GGCGTCGCCGATGTAGTGGG	CGTGCGCCTGCTGCATTTCC
6	4976249	SNP	T	C	GGTAGTCGGCCTTGATCTGT	GCACGAACGCAAGGAACTG
7	2772121	Insertion:1	GCCCCCCC	GCCCCCCCC	TCAACTCGCCCCGGCTGGTA	CGGTTGGCACCCTGGAGCAG
7	3874594	SNP	G	A	CCTACACGCCCCGGAAAC	CCGACCTACTTGCGCGAC
7	5314965	SNP	G	A	AATATCACCGACCACCTGCA	CTTGCCGAGTTCCTTGCCAA
8	784763	Insertion:1	TGGGGGGG	TGGGGGGGG	ACACCGTGAAAGAGCGCTG	CGTATTCCCAGAAATTCAGCGG
8	1023381	SNP	C	T	GTATCAACCTCGAGGCCTGG	GAAGACTTCCCGGGCGAC
8	1114264	Insertion:1	A	AG	CAAGCGCCGACCTCGACCTG	TCGCGCAGGTCGGTGGTGAA

Table S2 Genes that were mutated during big fitness drops. Core genes are indicated in bold.

Position	Reference	Alternative	Type of mutation	Effect	Gene ID	Gene description
Strain 2: day 4-6						
643143	**C**	**T**	**SNP**	**Non synonymous**	**PA0584**	**multifunctional tRNA nucleotidyl transferase/2'3'-cyclic phosphodiesterase/2'nucleotidase/ phosphatase**
2237340	A	G	SNP	Non synonymous	PA2044	Hypothetical protein
3224190	G	A	SNP	Non synonymous	PA2871	Hypothetical protein
4059231	G	A	SNP	Synonymous	PA3623	Hypothetical protein
4424181	G	A	SNP	Synonymous	PA3946	two-component sensor
4433030	GCC	GC	Deletion:1	Frameshift	PA3952	Hypothetical protein
4620706	G	A	SNP	Non synonymous	PA4131	iron-sulfur protein
5085288	CGGGGGG	CGGGGG	Deletion:1	Frameshift	PA4541	Hypothetical protein
5146358	GCCCCCCC	GCCCCCCCC	Insertion:1	Frameshift	PA4594	ABC transporter ATP-binding protein
Strain 5: day 14-16						
649784	**CGGGGGG**	**CGGGGGGG**	**Insertion:1**	**Frameshift**	**PA0590**	**diadenosine tetraphosphatase**
685295	T	TG	Insertion:1	Frameshift	PA0625	hypothetical protein
949677	G	A	SNP	Non synonymous	PA0868	peptidyl-tRNA hydrolase
2094973	C	T	SNP	Non synonymous	PA1920	anaerobic ribonucleoside triphosphate reductase
3464663	G	A	SNP	Non synonymous	PA3086	hypothetical protein
3912872	G	A	SNP	Non synonymous	PA3494	electron transport complex protein RsxE
3976134	A	G	SNP	Non synonymous	PA3549	alginate o-acetyltransferase AlgJ
4015416	**GCCCCCCC**	**GCCCCCCCC**	**Insertion:1**	**Frameshift**	**PA3583**	**glycerol-3-phosphate regulon repressor**
4747802	G	C	SNP	Synonymous	PA4233	major facilitator superfamily (MFS) transporter
5336256	**G**	**A**	**SNP**	**Synonymous**	**PA4751**	**cell division protein FtsH**
5824015	G	A	SNP	Synonymous	PA5172	ornithine carbamoyltransferase
Strain 6: day 0-2						
3270743	GCCCCCC	GCCCCC	Deletion:1	Frameshift	PA2914	ABC transporter permease
3572693	**G**	**A**	**SNP**	**Synonymous**	**PA3183**	**glucose-6-phosphate 1-dehydrogenase**
3856465	T	C	SNP	Synonymous	PA3451	hypothetical protein
4183909	**G**	**A**	**SNP**	**Non synonymous**	**PA3733**	**hypothetical protein**
4216781	T	C	SNP	Non synonymous	PA3763	phosphoribosylformylglycinamidine synthase
5465991	GGCGCGCGC	GGCGCGC	Deletion:2	Frameshift	PA4868	urease subunit alpha
6145798	G	A	SNP	Non synonymous	PA5455	hypothetical protein
Strain 6: day 2-4						
729307	**A**	**G**	**SNP**	**Non synonymous**	**PA0669**	**DNA polymerase subunit alpha**

949967	**A**	**G**	**SNP**	**Synonymous**	**PA0869**	**D-alanyl-D-alanine endopeptidase**
1727169	**A**	**G**	**SNP**	**Non synonymous**	**PA1586**	**dihydrolipoamide succinyltransferase**
2118685	TGGGGGGGG	TGGGGGGGG	Insertion:1	Frameshift	PA1937	hypothetical protein
5236051	**A**	**G**	**SNP**	**Non synonymous**	**PA4667**	**hypothetical protein**
5691426	**G**	**A**	**SNP**	**Non synonymous**	**PA5051**	**arginyl-tRNA synthetase**
5940774	**GCCCCCC**	**GCCCCCCC**	**Insertion:1**	**Frameshift**	**PA5275**	**frataxin-like protein**
Strain 7: day 20-22						
278627	A	G	SNP	Non synonymous	PA0246	major facilitator superfamily (MFS) transporter
1897501	**C**	**T**	**SNP**	**Non synonymous**	**PA1758**	**para-aminobenzoate synthase component I**
2627670	C	T	SNP	Synonymous	PA2376	transcriptional regulator
2645228	G	A	SNP	Synonymous	PA2390	protein PvdT
3384594	**G**	**A**	**SNP**	**Synonymous**	**PA3022**	**hypothetical protein**
3950587	**C**	**T**	**SNP**	**Non synonymous**	**PA3531**	**bacterioferritin**

Table S3 COG over- and under-enrichment analysis. Significance was calculated using Fisher's exact test and corrected for multiple testing using the false discovery rate correction method (BENJAMINI and HOCHBERG 1995).

COG Categories	N genes mutated	N genes in PAO1 genome	Fisher odds ratio	Fisher p-value	B-H correction
K (transcription)	28	390	0.530	0.001	0.023
G (carbohydrate transport and metabolism)	38	195	1.519	0.027	0.424
LK (replication, recombination and repair + transcription)	2	2	7.651	0.069	0.530
M (cell wall/membrane/envelope biogenesis)	39	224	1.351	0.093	0.530
KG (transcription + carbohydrate transport and metabolism)	2	3	5.100	0.106	0.530
C (energy production and conversion)	49	293	1.300	0.115	0.530
EH (amino acid transport and metabolism + coenzyme transport and metabolism)	6	21	2.192	0.120	0.530
CR (energy production and conversion + general function prediction)	3	9	2.552	0.154	0.597
H (coenzyme transport and metabolism)	15	164	0.691	0.192	0.662
N (cell motility)	1	28	0.272	0.245	0.736
EG (amino acid transport and metabolism + carbohydrate transport and metabolism)	1	2	3.820	0.309	0.736
ET (amino acid transport and metabolism + signal transduction mechanisms)	1	26	0.293	0.359	0.736
E (amino acid transport and metabolism)	59	399	1.142	0.360	0.736
MR (cell wall/membrane/envelope biogenesis + general function prediction)	1	3	2.547	0.389	0.736
CP (energy production and conversion + inorganic ion transport and metabolism)	1	3	2.543	0.389	0.736
IR (lipid transport and metabolism + general function prediction)	1	3	2.547	0.389	0.736
KT (transcription+ signal transduction mechanisms)	3	14	1.639	0.437	0.736
GER (carbohydrate transport and metabolism + amino acid transport and metabolism + general function prediction)	1	4	1.910	0.460	0.736
LR (replication, recombination and repair + general function prediction)	1	4	1.910	0.460	0.736
IQ (lipid transport and metabolism + secondary metabolites biosynthesis, transport and catabolism)	3	16	1.433	0.478	0.736
J (translation, ribosomal structure and biogenesis)	28	190	1.131	0.518	0.736
KL (transcription + replication, recombination and repair)	1	5	1.528	0.522	0.736
F (nucleotide transport and metabolism)	13	88	1.131	0.638	0.861
MU (cell wall/membrane/envelope biogenesis + intracellular trafficking, secretion and vesicular transport)	3	19	1.206	0.735	0.916

Linking Chapter 1 and 2

In Chapter 1 I performed a mutation accumulation experiment, resulting in several mutationally-degraded endpoint clones. Following on from this work, in Chapter 2 I evolved 4 of these clones in large populations to allow them to recover fitness. Together, these two chapters have allowed me to compare the evolutionary trajectories of populations founded by genotypes that differed by a large number of mostly-random mutations.

Chapter 2: The genomics of adaptation to mutational degradation in hypermutator *Pseudomonas aeruginosa*

Karl Heilbron, Danna R Gifford, and R. Craig MacLean

Department of Zoology, University of Oxford, Oxford OX1 3PS, United Kingdom

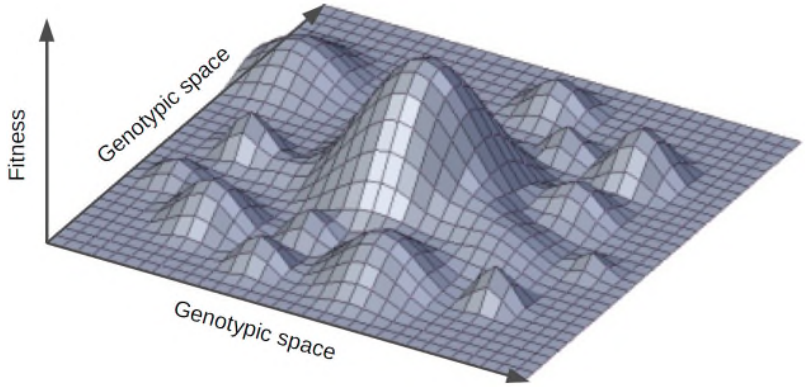
Abstract

The ruggedness of the fitness landscape influences whether genetically divergent populations will follow similar evolutionary paths when challenged with a common selective pressure. To study how variation impacts subsequent evolution, we introduced genetic divergence by propagating 4 mutation accumulation (MA) lines of hypermutator *Pseudomonas aeruginosa* for 644 generations (mean: 119 ± 33 SD random mutations per line). We founded 6 "recovery" populations from each MA line and allowed them to recover fitness for 70 generations by propagating them at a large effective population size. Genomic and fitness data from the recovery clones revealed several signatures of ruggedness, but only in lines with low post-MA fitness. First, recovery clones from the MA line with the lowest fitness exhibited impaired evolvability, possibly due to epistatic interactions that attenuated the effect of beneficial mutations. Second, in the two low-fitness MA lines there was a greater degree of convergence in the genes that were mutated when comparing recovery clones from the same MA line versus comparing clones from different MA lines. We examined parallel evolution in the *morA* gene in detail and found that *morA* mutations likely produced a larger epistatic benefit in clones from one low-fitness MA line, possibly by decreasing motility. These results suggest that, while neutral genetic diversity may do little to alter the routes of adaptation taken by evolving populations, acquiring deleterious mutations may cause low-fitness populations to experience more rugged fitness landscapes.

Introduction

Genetic diversity arises in even the simplest of environments (TREVES *et al.* 1998; ROZEN *et al.* 2009). Adaptive evolution requires the fixation of beneficial mutations, but non-additive interactions between incoming mutations and the genetic diversity in the population (called "epistasis") may cause the different genotypes in a population to take divergent mutational paths to higher fitness.

Evolutionary biologists have long been interested in whether evolving populations are frequently faced with forks-in-the-road during adaptive walks—two or more alternative mutational pathways to higher fitness that cannot be combined. Sewell Wright (WRIGHT 1932) visualized evolution as a hill-climbing process in a three-dimensional landscape where different genotypes are arranged by genetic similarity to one another on the horizontal axes and the fitness of each genotype is plotted on the vertical axis (Supplementary Figure 1). Here, forks-in-the-road create "rugged" fitness landscapes with multiple peaks, and evolving populations may become trapped upon lesser peaks. If ruggedness is a common feature of fitness landscapes then the ultimate outcome of which peak a population ends up on may be influenced by epistatic interactions between genetic diversity in a population and incoming mutations.



Supplementary Figure 1 An example of a rugged three-dimensional fitness landscape. The presence of multiple peaks could cause a population evolving on this landscape to become stuck on a lesser peak, unable to reach maximum possible fitness in the landscape. This figure is reproduced under the Creative Commons Attribution-Noncommercial license from http://cairnarvon.rotahall.org/2007/01/02/on-fitness-landscapes/.

One way to explore the structure of the fitness landscape is to apply experimental evolution to populations founded by genotypes that differ from each other by a number of mutations (ESTES AND LYNCH 2003; MOORE AND WOODS 2006; BARRICK *et al.* 2010; MACLEAN *et al.* 2010b; GIFFORD *et al.* 2011; KRYAZHIMSKIY *et al.* 2014; PERFEITO *et al.* 2014). A prevailing trend in these studies has been that the starting fitness of a genotype plays a large role in evolvability—the change in fitness during the experiment. Typically, the less fit the initial genotype is, the greater its increase in fitness during evolution, causing the fitness of the different evolving genotypes to converge over time. The actual mutations in those genotypes, however, may matter less.

By combining evolution experiments founded by different genotypes and whole genome re-sequencing, it is possible to determine whether the different genotypes evolve via similar genetic mechanisms. Kryazhimskiy *et al.* (KRYAZHIMSKIY *et al.* 2014) sequenced 104 genomes from 13 laboratory-evolved yeast populations that initially differed by 5-6 mutations. They found that initial mutations had little impact on evolvability and therefore concluded that their populations had evolved on a relatively smooth fitness landscape. Recent work has suggested that fitness landscapes may be more rugged when populations are farther from a

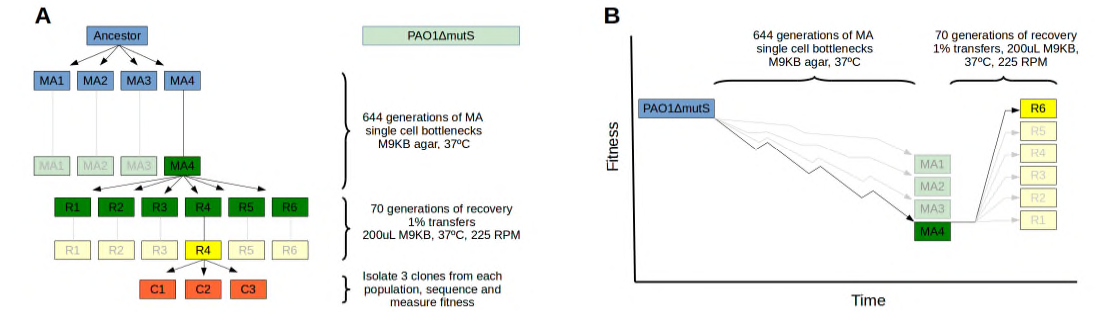
fitness peak (Supplementary Figure 1) (KRYAZHIMSKIY *et al.* 2012; NAHUM *et al.* 2015). However, no study has yet explored the genomics of adaptation in genotypes that have been more broadly spread across the fitness landscape by a greater number of mutations. One set of natural systems in which this may be particularly relevant is in species of bacterial endosymbionts that live in small, frequently bottlenecked populations (MIRA AND MORAN 2002) and therefore diverge rapidly due to the fixation of mutations due to drift (MORAN *et al.* 2009).

To investigate the outcome of evolution in strains separated by a greater genetic distance we performed replicate evolution experiments founded from strains that had diverged by an average of 119 mutations due to ~644 generations of nearly random mutation accumulation. We found several signatures of evolution on a rugged fitness landscape in these populations, particularly in those with low levels of post-MA fitness.

Materials and Methods

Strains

In a previous study (HEILBRON *et al.* 2014), we propagated 8 mutation accumulation lines of hypermutator *P. aeruginosa* at an effective population size (N_e) of ~16 individuals for ~644 generations. We selected the end points of 4 of these MA lines where fitness did not become so low that it was difficult to measure accurately (Supplementary Figure 2). Each end point clone contained an average of 119 (±33 SD) random mutations. In this study these lines are named Line A-D in ascending order of post-MA fitness and respectively correspond to MA line 8, 4, 1, and 3 from the previous study. An ancestral PAO1-GFP normomutator (*i.e.* wild-type mutation rate) was used as a reference strain in competitive fitness assays (HEILBRON *et al.* 2014).



Supplementary Figure 2 Schematic depicting the experimental design of this study. **(A)** The ancestral Δ*mutS* PAO1 strain was split into 4 MA lines (MA1 - MA4), each of which was propagated for 644 generations at a small effective population size. Each MA line endpoint was split into 6 "recovery lines" (R1 – R6) and each was propagated for 70 generations at a large effective population size. For each recovery line end point, 3 clones were sequenced and assayed for fitness. **(B)** An alternative representation of the layout presented in (A) that depicts how fitness is expected to change over time as a result of MA and recovery.

Fitness recovery

Frozen stocks of the 4 MA endpoints were streaked on M9KB agar and 6 colonies were randomly picked from each to generate replicate "recovery lines" (Supplementary Figure 2). A further 7 "control lines" were established from the ancestral hypermutator strain that did not undergo MA. Recovery and control lines were inoculated into 200µL M9KB broth at 37°C and each day 2µL was transferred to fresh medium, representing a 100-fold dilution (~6.6 generations/transfer). Populations were frozen at −80°C daily in 50% glycerol for 10 days. At the end of the experiment, populations were streaked on M9KB agar plates and three random clones were selected and frozen in 50% glycerol.

Competitive fitness assays

Fitness relative to the PAO1-GFP strain was measured for the starting population (day 1) and 3 end point clones (day 10) of all 24 recovery lines and all 7 control lines, using previously described methods (HEILBRON *et al.* 2014). Briefly, 50:50 mixtures of the strains were competed in the same environment as the fitness recovery experiment: ~22 hours at 37°C with 200 RPM shaking. The initial and final proportions of each competing strain was determined by

flow cytometry and fitness was calculated using the formula:

$$w_{\text{mutant}} = \frac{\log_2\left(\frac{N_{\text{final, mutant}}}{N_{\text{initial, mutant}}}\right)}{\log_2\left(\frac{N_{\text{final, wild type}}}{N_{\text{initial, wild type}}}\right)}$$

where w_{mutant} is the fitness of the mutant relative to the wild-type and N_{ij} is the number of either the mutant or the wild-type cells at either the beginning or the end of the competition. All fitnesses are reported relative to the average initial fitness of the unmutated ancestral strain. Three biological replicates of three technical replicates each were performed for each strain.

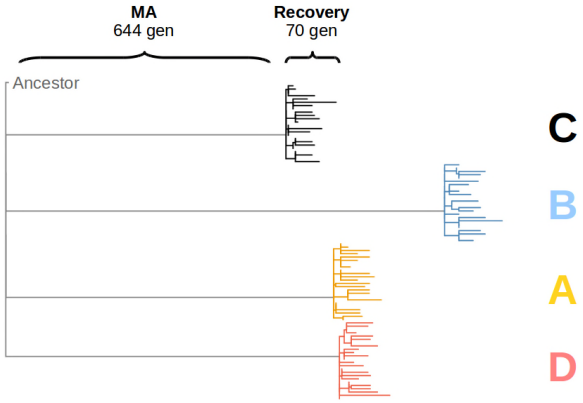
Whole genome sequencing and variant calling

We performed 100bp paired-end Illumina HiSeq2000 whole genome re-sequencing on all 96 recovery populations and clones for which fitness was determined. Reads were mapped to the PAO1 reference genome (NC_002516.2) and variant calling was performed as in Heilbron *et al.* (HEILBRON *et al.* 2014) (see Supplementary Material for further information). Genomic positions that were mutated more than once independently, mostly commonly due to expansion and contraction of homopolymeric tracts (*i.e.* stretches of DNA where a single nucleotide is repeated multiple times), were analyzed separately from the main analysis.

Phylogeny

We generated a maximum likelihood phylogenetic SNP tree with GARLIv0.951 (ZWICKL 2006) using a general time reversible model of nucleotide substitution with gamma distributed rate heterogeneity, six rate categories, and a proportion of invariant sites (Supplementary Figure 3). We ran the program ten times using different random starting trees to ensure

convergence. There was no evidence of cross-contamination: all samples clustered according to their respective MA line and recovery line. Because there was little phylogenetic structure in our samples we did not correct for phylogeny in our subsequent analyses.



Supplementary Figure 3 Phylogeny of sequenced recovery clones. Samples cluster by MA line and are named A – D in ascending order of post-MA fitness. All samples from the same recovery line form monophyletic groups.

Repeatedly-mutated genes and intergenic regions

"Repeatedly-mutated genes" (RMGs) were defined as genes mutated ≥3 times independently in the recovery or control lines, excluding synonymous mutations. We used simulations to determine this threshold by randomly allocating the same number of mutations as was observed in this study into a simulated genome using the actual distribution of *P. aeruginosa* gene lengths. This was repeated 10,000 times and the results were averaged across simulations (mean number of mutations per gene due to chance = 0.1). We performed the same analysis on intergenic regions, but used a less stringent cutoff of ≥2 independent mutations as our criterion for parallel evolution because intergenic regions are ~7x shorter than genes, on average (mean intergenic: 145bp, mean gene: 1002bp, mean number of mutations per intergenic region due to chance = 0.03).

Motility assays

To assay motility we picked individual colonies from each strain, stabbed them into the centre of M9KB soft agar plates (0.25% agar), and incubated for 18 hours at 37C. The following day we recorded the maximum diameter of bacterial growth.

Statistics

All statistical analyses were conducted using R version 3.2.2 (R CORE TEAM 2015). Degrees of freedom are reported as subscripts next to the test statistic. All tests use $\alpha = 0.05$ and are two-tailed, where applicable.

Results

Experimental overview

The goal of our study was to determine the outcome of evolution within and between strains of Δ*mutS* hypermutator *P. aeruginosa* that had diverged by a substantial number of random mutations. Divergence was achieved by propagating 8 MA lines through single-cell bottlenecks for ~644 generations to minimize the influence of natural selection (HEILBRON *et al.* 2014). On average, each MA line accumulated 118 mutations (± 23 SD) with no significant difference in the number of mutations accumulated between the MA lines (one-sample Kolmogorov–Smirnoff test: $P = 0.521$, $D = 0.270$). To study fitness recovery, we selected endpoint clones from 4 MA lines where fitness did not become so low that it was difficult to measure accurately (Supplementary Figure 2). To allow fitness to recover, we evolved 6 replicate "recovery" populations founded from each of these MA lines for 70 generations in large populations. We also propagated 7 "control" populations that did not undergo previous mutation accumulation. To explore the genomics of adaptation, we sequenced the starting population and three endpoint clones from each recovery and control.

Evolvability

Prior to recovery, the 4 MA endpoint strains spanned a wide range of initial fitness values (0.64 – 1.16). Hereafter we will refer to the 4 MA lines as Line A – D in ascending order of initial fitness. If the recovery populations evolved on a smooth fitness landscape, then recovery clones from different MA lines should have evolved towards similar levels of post-recovery fitness. Data from the three MA lines with the greatest initial fitnesses (Lines B, C, and D) supported this hypothesis: the lower the initial fitness of an MA line, the greater its evolvability (defined as "change in fitness during recovery"; Figure 1). On average, this caused clones from lower-fitness MA lines to approach but not exceed the fitness of clones from higher-fitness MA lines. However, the lineage with the lowest initial fitness, Line A, exhibited impaired evolvability with endpoint recovery clones only reaching an average final fitness of 0.70 (±0.02 SE). This is suggestive of ruggedness in the fitness landscape experienced by Line A recoveries. It is possible that Line A recoveries have become stuck on a lesser fitness peak, but these populations would need to undergo a longer duration of experimental evolution to properly test this hypothesis.

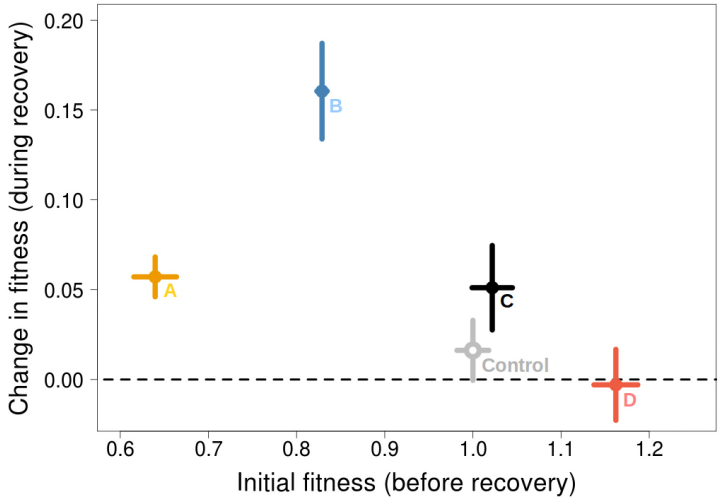


Figure 1 The effect of pre-recovery fitness on evolvability. Data are presented as averages (± SE) for all end point clones (evolvability) or initial populations (initial fitness), separated by MA line or control. The dashed line represents no change in fitness during 70 generations of recovery evolution.

Evolution of the mutation rate

The decreased evolvability of Line A recovery clones may have resulted from a decreased mutation rate in Line A. In order to test this hypothesis we sequenced all endpoint clones and their founding populations. A total of 724 mutations fixed during recovery across all recovery lines with an average of 10.1 (± 4.7 SD) mutations per endpoint clone. The three end point clones from each recovery tended to diverge early on in the experiment, sharing an average of only 0.35 mutations (see Supplementary Figure 3 for the phylogeny). There was no significant difference in the number of independent mutations acquired in recovery populations across MA lines (ANOVA: P= 0.232, $F_{3,20}$= 1.552), indicating that impaired evolvability in Line A recovery clones was not due to an altered mutation rate.

The genomics of adaptation to mutational degradation

Next, we investigated the genomic data for molecular signatures of evolution on a rugged fitness landscape. Because the mutation rate of the hypermutator strain used in this study is ~70x greater than the wild-type, many of the acquired mutations are expected to be neutral hitchhikers. To disentangle beneficial mutations from neutral hitchhikers, we focused on the 9 genes that were mutated ≥3 times independently in the recovery or control lines ("repeatedly-mutated genes" or RMGs). Of the 30 recovery mutations in RMGs (4.3% of all mutations): 25 were non-synonymous, 4 were frameshifts, and 1 mutation created a premature stop codon, but there were no synonymous mutations. Because of the absence of synonymous mutations it was not possible to calculate dN/dS, but given a total of 30 mutations we would have expected 6.7 synonymous mutations due to chance.

While there was no significant relationship between evolvability and the total number of mutations borne by endpoint clones (ANOVA: P= 0.444, $F_{1,67}$= 0.593), acquiring RMG mutations caused a significant increase in fitness (Figure 2; ANOVA: P< 0.001, $F_{1,67}$= 16.216). "Repeatedly-mutated intergenic regions", did not have a significant effect on evolvability

(ANOVA: P= 0.342, $F_{1,66}$= 0.915) and so were dropped from the analysis. Of the 9 RMGs, 7 are involved in the production of energetically expensive products that are not needed for growth in well-mixed laboratory environments, such as the production of iron-scavenging molecules, virulence factors, and flagella (Table 1). The 2 other RMGs are involved in non-homologous DNA repair (*ligD*) and the formation of iron-sulphur clusters (*ydiU*).

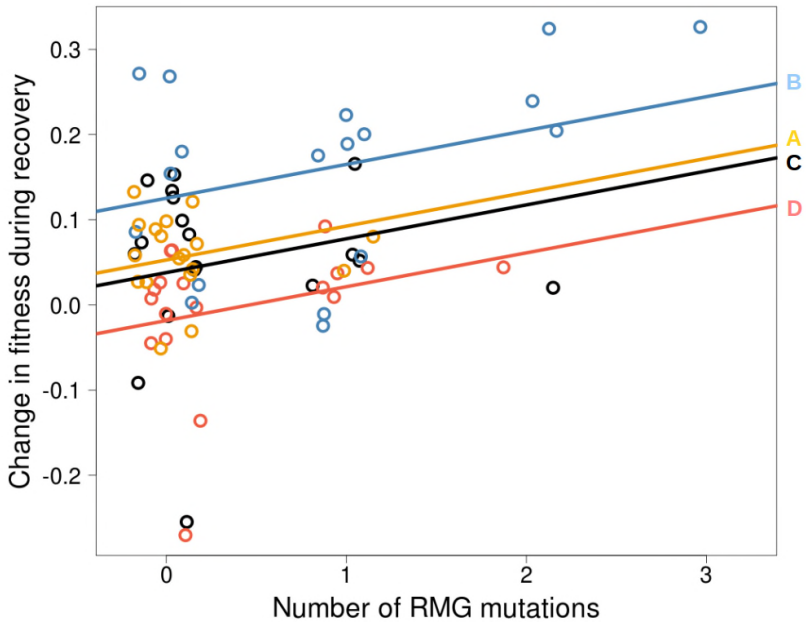


Figure 2 Mutations in repeatedly-mutated genes (RMGs) caused a significant increase in fitness (P< 0.001). Lines represent the fit of a linear model where evolvability is explained by the number of RMG mutations carried by a clone (numerical) and by MA line (categorical) with no interaction term (therefore all lines have the same slope). Data on the x-axis data has been displaced slightly for clarity.

In addition to these RMGs, we were interested in searching for other types of mutations that contributed to fitness gain. In the original MA experiment (HEILBRON *et al.* 2014) we found that 95.3% of frameshift mutations were in hypermutable homopolymeric tracts—stretches of DNA where the same nucleotide is found 4-8 times in a row. Since we also found evidence of strong purifying selection against frameshifts, we hypothesized that reversion of frameshifts acquired during MA in these still-hypermutable regions would be involved in fitness recovery. However, we did not find a significant relationship between the number of

reversion mutations borne by an endpoint clone and its evolvability (ANOVA: P= 0.335, $F_{1,66}$= 0.943), suggesting that the frameshift mutations that did manage to become fixed during MA were not strongly deleterious.

Table 1 List of repeatedly-mutated genes and their functions

Locus tag	Gene	Function
PA0625	–	R-type pyocin (LLAMAS *et al.* 2008)
PA1430	*lasR*	Transcriptional regulator involved in quorum sensing
PA2138	*ligD*	Multifunctional non-homologous end joining protein
PA2402	–	Probably involved in pyoverdine synthesis
PA2424	*pvdL*	Catalyzes a crucial step in pyoverdine synthesis
PA4226	*pchE*	Essential for pyochelin synthesis
PA4541	*lepA*	Extracellular protease
PA4601	*morA*	Motility regulator
PA5023	*ydiU*	Involved in iron-sulphur cluster formation

Impaired evolvability in Line A

If recovery populations evolved on a smooth fitness landscape, then recovery clones from the different MA lines should have acquired approximately the same number of mutations in RMGs. This was not the case: the distribution of RMG mutations was not random across the 4 MA lines (Figure 3; Poisson GLM: P= 0.004, $Deviance_{3,68}$= 13.282). Interestingly, clones from the line with reduced evolvability, Line A, also acquired the fewest RMG mutations. Line A clones did not significantly differ from the other lines with respect to their mutation rate (ANOVA: P= 0.232, $F_{3,20}$= 1.552); rather, the paucity of RMG mutations in Line A

recovery clones may have been due to epistatic interactions with MA mutations in Line A that attenuated or destroyed the benefit conferred by these RMG mutations. Because Line A accumulated 99 mutations during the MA experiment it is difficult to disentangle which mutation or mutations could be responsible for its impaired evolvability. Large deletions that remove one or more genes could irreversibly impair evolvability; however, all deletions in the MA lines used in this study only removed a single nucleotide. Thus, the source of Line A's reduced evolvability is uncertain.

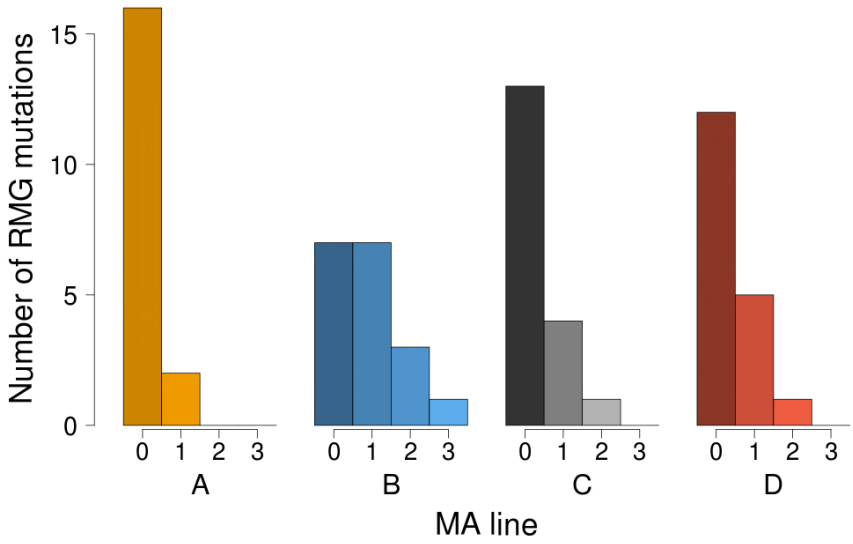


Figure 3 Distribution of repeatedly-mutated genes by MA line. Recovery clones acquired between 0 – 3 mutations in repeatedly-genes. Recovery clones from different MA lines differed significantly in the number of RMG mutations that they acquired (*P*= 0.004) and clones from the least fit MA line, Line A, acquired the fewest RMG mutations.

Genetic convergence within and between MA lines

In a smooth fitness landscape different genotypes are all located within the domain of attraction of a single fitness peak and are expected to fix mutations in similar genes. On the other hand, in rugged fitness landscapes different genotypes may reside in the domain of attraction of different fitness peaks. In the latter case, multiple populations founded from the same genotype are expected to fix more similar mutations to one another than to the mutations fixed in populations founded from other genotypes.

Although RMGs were independently mutated several times during the recovery phase, there was little convergence in the genes that were hit in different endpoint recovery clones. On average, pairs of clones only had 0.047 genes mutated in common ("shared mutated genes"). Pairs of recovery clones from the same MA line shared ~3x more mutated genes than pairs of clones from different MA lines (0.096 v. 0.033 mutated genes; Poisson GLM: $P< 0.001$, $Deviance_1= 59.654$). Therefore, evolution proceeded slightly differently in recoveries founded from the different MA lines, suggesting a degree of ruggedness in the fitness landscape. Furthermore, this trend was not consistent across MA lines (Figure 4). Recoveries from the two low-fitness MA lines, Lines A and B, had 4.8x and 6.4x more within-line shared mutated genes than between-line shared mutated genes, respectively (Poisson GLMs, A: $P <0.001$, B: $P <0.001$). On the other hand, in Lines C and D the number of within-line shared mutated genes was not significantly different from the number of between-line shared mutated genes (Poisson GLMs, C: $P= 0.074$, D: $P= 0.177$). These results suggest that the fitness landscape is more rugged for recoveries from the two low-fitness MA lines than it is for those from the two high-fitness MA lines.

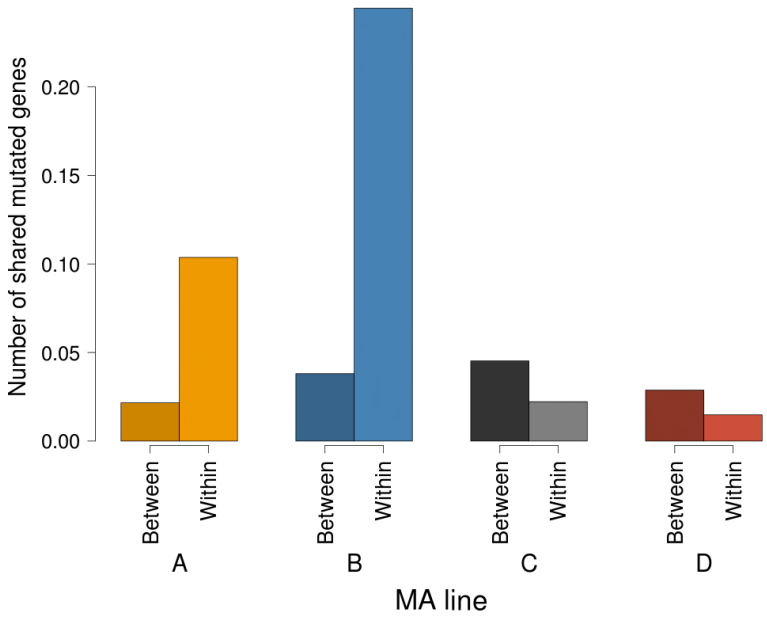


Figure 4 The average number of shared mutated genes ("convergence") in pairs of recovery clones from the same MA line (within) or different MA lines (between). Convergence is greater within-line for the two low-fitness MA lines (A: P <0.001, B: P <0.001). There is no significant difference in within- and between-line convergence for the two high-fitness MA lines (C: P= 0.074, D: P= 0.177).

Example of genetic convergence: morA

The most repeatedly-hit gene across all recoveries, *morA*, displayed a particularly strong signature of increased within-line convergence so we investigated it in further detail. *morA* was the only RMG that was significantly non-randomly distributed across the 4 MA lines—8 of the 10 *morA* mutations were in recoveries founded from Line B (FDR-corrected χ^2 test with a simulated null distribution: P< 0.001, χ_4= 10.286). Line B was also the only line that acquired a mutation in *morA* during the MA phase of the experiment, suggesting that this MA *morA* mutation may have potentiated future recovery mutations in Line B. *morA* controls cellular levels of cyclic di-GMP (c-di-GMP), which in turn regulates motility, virulence factor production, and biofilm formation. We performed motility assays on pairs of closely-related recovery clones that had or had not acquired a *morA* mutation and found that fixing a *morA* mutation during recovery led to a significant reduction in motility (paired t-test: P= 0.020, t_8= -2.886). Recovery clones with *morA* mutations were 11% fitter than those without (two-sample

t-test: P= 0.034, t_{16}= -2.311), suggesting that this fitness gain may have been due to reduced motility in a well-mixed environment. The average fitness increase in Line B endpoint clones was 16%, so *morA* mutations accounted for a large fraction this fitness gain.

The *morA* gene contains 5 functional domains that collectively comprise 52% of the gene length. However, all 8 *morA* SNPs were in (7 SNPs) or within 14 nucleotides of (1 SNPs) these domains (binomial test: P= 0.008). In addition, there were two independent frameshift mutations at the same site near the start of the gene, likely completely knocking out *morA* function. Two of the *morA* domains are involved in the synthesis (diguanylate cyclase domain) and degradation (phosphodiesterase domain) of c-di-GMP, a secondary messenger molecule that regulates motility, virulence factor production, and biofilm formation. However, only 2 of the 10 *morA* mutations were in either of these domains, so *morA* mutations outside of these two domains can also modulate motility.

Intragenic compensatory evolution

Because we found that the enrichment of Line B recovery mutations in *morA* may have been due to a Line B MA mutation in *morA*, we tested whether this phenomenon occurred more broadly. Specifically, for each line we were interested in whether genes that were mutated during MA were more likely to acquire a second recovery mutation in those same genes (hereafter referred to as "putative intragenic compensatory mutations"), possibly to ameliorate defects caused by the original mutations. However, the average number of recovery mutations in a gene was not related to whether that gene was mutated during MA or not (sign test: P= 0.434). Furthermore, there was no association between evolvability and the number of putative intragenic compensatory mutations borne by a recovery clone (ANOVA: P= 0.824, $F_{1,66}$= 0.050). We therefore concluded that intragenic compensation was not a significant driver of adaptation to mutational degradation despite the large number of MA mutations fixed in protein-coding genes in each MA line.

Discussion

In this paper we were interested in studying the ruggedness of fitness landscapes experienced by evolving populations founded from genotypes that differed by a large number of mutations. We found three lines of evidence that support the idea that fitness landscapes may appear more rugged in poorly-adapted populations (KRYAZHIMSKIY *et al.* 2012; NAHUM *et al.* 2015). First, recovery clones from the MA line with the lowest fitness had an impaired capacity to evolve given the conditions and time frame of this experiment. Second, there was greater parallelism in pairs of recovery clones from within the same MA line than in pairs from different MA lines, but only for clones from the two low-fitness MA lines. Finally, recovery mutations in *morA* were significantly enriched in clones from Line B, probably due to the acquisition of a *morA* mutation during MA in Line B.

Impaired evolvability

Despite much recent work that has shown that less-fit genotypes tend to be more evolvable (ESTES AND LYNCH 2003; MOORE AND WOODS 2006; BARRICK *et al.* 2010; MACLEAN *et al.* 2010b; GIFFORD *et al.* 2011; KRYAZHIMSKIY *et al.* 2014; PERFEITO *et al.* 2014), recovery clones from the MA line with the lowest fitness, Line A, only showed marginal increases in fitness during this experiment. This was not due to a decrease in mutation rate, but rather may have been due to epistatic interactions that reduced or eliminated the selective benefit of mutations that were beneficial in the other MA lines. Because Line A acquired 99 mutations during MA we were unable to determine which mutation(s) were responsible for this impaired evolvability, but previous studies have shown that it is possible for evolvability to be dramatically altered by a single SNP (HALL *et al.* 2010; KHAN *et al.* 2011; GIFFORD AND MACLEAN 2013). More distantly, impaired evolvability has been observed after the addition of a novel biochemical pathway into different species of *Methylobacterium* (MICHENER *et al.* 2014).

It is possible that if we had run the recovery phase of this experiment longer, then Line

A recovery clones may have been able to overcome their initial disadvantage. Kryazhimskiy and colleagues (KRYAZHIMSKIY *et al.* 2014) found that when their yeast populations were evolved for 500 generations instead of 250 generations, the predictability of evolvability became more strongly influenced by the initial fitness of the genotypes used in the study and less influenced by the specific mutations harboured by those genotypes. Another possible reason why Line A displayed reduced evolvability is that it had a CFP gene engineered into its mini-Tn7 site prior to MA while the other three lines did not, however, this is unlikely. We compared the evolvability of unmutated ancestors with and without the CFP tag and found no significant difference (ANOVA: P= 0.651, $F_{1,22}$= 0.211). Furthermore, several previous studies have shown that mini-Tn7 integrations have no effect on fitness (CHOI *et al.* 2005; MAVRODI *et al.* 2011).

Compensatory evolution

Compensatory mutations are expected to be readily available in highly-mutated genetic backgrounds such as those used in this study (SILANDER *et al.* 2007). For every deleterious mutation there are estimated to be ~12 compensatory mutations that become available (POON *et al.* 2005) and therefore compensatory mutations are thought to dominate recovery evolution (SCHRAG AND PERROT 1996; REYNOLDS 2000; MAISNIER-PATIN *et al.* 2002; BRANDIS *et al.* 2012). Consistent with these expectations we found approximately three-fold greater convergence in recovery clones from the same MA line than for recovery clones from different MA lines. Furthermore, this effect was entirely driven by the two low-fitness MA lines. This suggests that the unique mutations that separate the two low-fitness MA lines caused their recoveries to proceed via slightly different routes.

It is difficult to estimate how much of recovery evolution was due to compensatory mutation and how much was due to generally beneficial mutations. Very few of the mutations found in endpoint recovery clones are expected to be beneficial (via either mechanism)

because of the short duration of the recovery experiment (70 generations) and the hypermutator nature of the strains used. Compensatory mutations may have only played a minor role due to their rarity—about 1 shared mutated gene for every 21 pairs of endpoint clones. However, shared mutated genes only represent one type of compensatory mutation. These analyses will miss cases where recovery clones from the same MA line evolve more similarly to one another due to mutations in different genes that affect a common phenotype.

Approximately 92% of compensatory mutations are estimated to be intragenic in prokaryotes (POON *et al.* 2005), but we found no evidence of an elevated rate of intragenic compensatory mutation, or that putative intragenic compensatory mutations caused an increase in fitness. We did find that mutations in *morA* exhibited a hallmark of intragenic compensation: mutations were significantly enriched in Line B, the only line that had acquired a *morA* mutation during MA. These mutations caused an increase in fitness of ~11%, possibly by decreasing motility (motility is unnecessary in the well-mixed laboratory environment in which these populations were evolved).

However, *morA* mutations do not strictly fit the definition of a compensatory mutation, which must be neutral or deleterious in the absence of the mutation for which it is compensating (KIMURA 1985). Two of the ten *morA* mutations in this experiment were in lines that did not acquire a predisposing *morA* mutation during MA. This suggests that *morA* mutations are beneficial across genetic backgrounds, but engendered a much greater fitness benefit in Line B, a phenomenon called “magnitude epistasis”.

The paucity of intragenic compensatory mutations in this study was surprising, although this was also found to be the case in a similar study in *Caenorhabditis elegans* (DENVER *et al.* 2010). Given the short duration of our bacterial selection experiment (70 generations), only a small number of beneficial mutations are expected to be present in each recovery clone. These beneficial mutations therefore represent the first steps in an adaptive walk, and are expected to confer a large fitness benefit relative to the total pool of beneficial

mutations (ORR 2002). Since the vast majority of deleterious MA mutations only had very small fitness consequences (HEILBRON *et al.* 2014), the lack of intragenic compensatory mutations may have been because they would only have provided marginal fitness increases and were outcompeted by mutations of larger effect. As such, the component of fitness loss that was due to small-effect mutations during MA may be very difficult to recover from, a phenomenon which has been observed in synthetically engineered polioviruses (COLEMAN *et al.* 2008).

Motility

In our hypermutator strain of *P. aeruginosa* PAO1 we found that *morA* mutations led to decreased motility and increased fitness. In contrast, a previous study found that *morA* regulated motility in *Pseudomonas putida*, but not in *P. aeruginosa* PAO1 (CHOY *et al.* 2004). However, *P. aeruginosa* PAO1 isolates from around the world differ greatly both phenotypically (including differences in their survival under nutrient deficiency and *in vivo* virulence) and genotypically (including prophage duplications, up to 1kb deletions, and a 2.2Mb inversion) (KLOCKGETHER *et al.* 2010). Thus, the ability of *morA* to regulate motility depends upon the strain of *P. aeruginosa* PAO1 under study, underscoring the difficulty of drawing general conclusions about a species from studies of a single strain.

Positive selection during MA

Although the small effective population size of the MA experiment decreased the efficacy of selection (HEILBRON *et al.* 2014), selection was not entirely removed. In fact, across all 8 MA lines from the original experiment there were 12 RMG mutations. There are two explanations for this phenomenon: 1) RMGs are hypermutable, causing them to acquire mutations regardless of the strength of natural selection; or 2) RMG mutations are sufficiently beneficial to be fixed under MA conditions. The gene PA2402 may be hypermutable as 3/3 PA2402 MA mutations were synonymous (although there is a growing body of examples of

synonymous mutations with non-zero fitness consequences, e.g. (BAILEY *et al.* 2014)). On the other hand, only 1 of the 9 remaining RMG mutations from the 8 original MA lines was synonymous, suggesting that these were under positive selection as well.

Conclusion

Although it is convenient to depict fitness landscapes as three-dimensional, in reality the dimensionality of genotype spaces is vastly larger. Studies have begun to probe small regions of this immense space and are beginning to shed light on the topology of fitness landscapes at different scales. At the smallest scale, sign epistasis between pairs of mutations creates local ruggedness. At a medium scale, however, the genetic diversity between closely related genotypes may do little to constrain the outcome of evolution, suggesting a smoothing of the fitness landscape. The main contribution of our study is to provide evidence to support recent arguments that fitness landscapes once again become more rugged at larger scales. Because we find that this effect is driven by recovery clones from low-fitness MA lines, future work will need to disentangle the extent to which ruggedness is caused by decreased fitness versus increased mutation accumulation. Another key question that will need to be addressed is how ruggedness is affected at even larger scales, such as between different strains or different species.

Acknowledgements

We would like to thank Alvaro San Millan for his insightful comments throughout the project. This work was supported by funding from The Royal Society (RCM), the Natural Sciences and Engineering Research Council of Canada (KH), the Clarendon Fund (KH), and The Queen's College, Oxford (KH). We thank the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics (funded by Wellcome Trust grant 090532/Z/09/Z and Medical Research Council hub grant G0900747 91070) for generating the sequencing data.

Supplementary Material

Whole genome sequencing and variant calling

The whole genome sequencing analysis was performed as described in San Millan *et al.* (SAN MILLAN *et al.* 2014). Whole genome sequencing services were provided by the Wellcome Trust Centre for Human Genetics (Oxford, UK) using the Illumina HiSeq2000 platform with 100 bp paired-end reads. Initial read filtering was done using NIH QC Toolkit (PATEL AND JAIN 2012). 5' or 3' ends were trimmed if the Phred quality score was less than 20. Reads were discarded if they were shorter than 50 bp after trimming, if more than 2% of bases were ambiguous, or if more than 20% of bases had a Phred score < 20. BWA was used to map filtered reads to the *P. aeruginosa* PAO1 reference genome (NC_002516.2). Mapped reads were processed to increase the quality of the variant calling: 1) reads with multiple best hits were discarded; 2) duplicated reads were discarded using MarkDuplicates from the Picard package (http://picard.sourceforge.net); 3) reads around indels were locally realigned using RealignerTargetCreator and IndelRealigner from the GATK package to correct for misalignment; and 4) mate pairs were sorted using FixMateInformation in the Picard package. Variant calling was performed with GATK's Unified Genotyper (DEPRISTO *et al.* 2011) and Samtools's mpileup (LI *et al.* 2009). VCFtools (vcf-annotate, (DANECEK *et al.* 2011)) and GATK toolkit (VariantFiltration, (DEPRISTO *et al.* 2011)) were used to filter the raw variants for strand bias, end distance bias, base quality bias, SNPs around gaps, low coverage and erroneously high coverage. Variants were combined using GATK's CombineVariants (keeping any unfiltered). High quality variants not filtered were annotated using SnpEff (CINGOLANI *et al.* 2012).

Three approaches were used to detect structural variants. First, BreakDancer (CHEN *et al.* 2009) was used to predict deletions, insertions, inversions, and translocations using deviations in the separation or orientation of mapped read pairs. Pindel (YE *et al.* 2009) was used to infer deletions, short insertions, long insertions, inversions, tandem duplications, and

breakpoints using a split-read approach (the output of BreakDancer was also fed to Pindel to improve its output). Finally, Control-FREEC (BOEVA *et al.* 2012) was used to detect copy number variants (CNVs). Control-FREEC finds CNVs using depth-of-coverage (normalized by GC-content). Regions of low mappability were excluded by supplying Control-FREEC with mappability tracks generated by gem-mappability (GEM library, (MARCO-SOLA *et al.* 2012)).

Linking Chapter 2 and 3

In Chapter 2 I investigated the adaptive landscape in populations founded from genotypes that differed by a large number of mutations. One interesting result that arose from this work was that the initial fitness of these genotypes may play a role in the ruggedness of the landscape, altering their ability to adapt to their environment. Following on from this work, in Chapter 3 I measured how the fitness gain engendered by a beneficial mutation was affected by the initial fitness of its genetic background in all pairwise combinations of three beneficial mutations and three deleterious mutations. Furthermore, I tested whether the cost of these deleterious mutations was associated with initial fitness.

Chapter 3: Adaptation to a novel environment can decrease the cost of rifampicin resistance in *Pseudomonas aeruginosa*

Karl Heilbron, Qin Qi, and R. Craig MacLean

Department of Zoology, University of Oxford, Oxford OX1 3PS, United Kingdom

Abstract

Antibiotic resistance is a growing global problem that threatens to undermine our ability to treat life-threatening bacterial infections. Mutations that increase antibiotic resistance usually result in decreased fitness in antibiotic-free environments and this "cost of resistance" is thought to be one of the main determinants of the spread and maintenance of resistance. From an evolutionary standpoint, little is known about how the fitness of an organism affects its ability to acquire costly antibiotic resistance mechanisms. Here, we compared the fitness costs associated with antibiotic resistance mutations in genetic backgrounds that differed in fitness. We evolved populations of *Pseudomonas aeruginosa* under laboratory conditions and used whole genome sequencing to identify three clones that contained 1-2 beneficial mutations, each representing a different step along an adaptive walk. We then introduced three costly rifampicin resistance mutations into each evolved background, as well as into the wild-type, and measured fitness in the absence of antibiotics. We found no relationship between the cost of a resistance mutation and the fitness of the genetic background into which it was introduced. However, we found significant positive epistasis between the beneficial and resistance mutations, resulting in the resistance mutations engendering a lesser fitness cost in genetic backgrounds carrying beneficial mutations versus the wild-type background. These results show that it is possible for

adaptation to a novel environment to lead to a decrease in the cost of acquiring antibiotic resistance, but this phenomenon is not predictable from the initial fitness of that background.

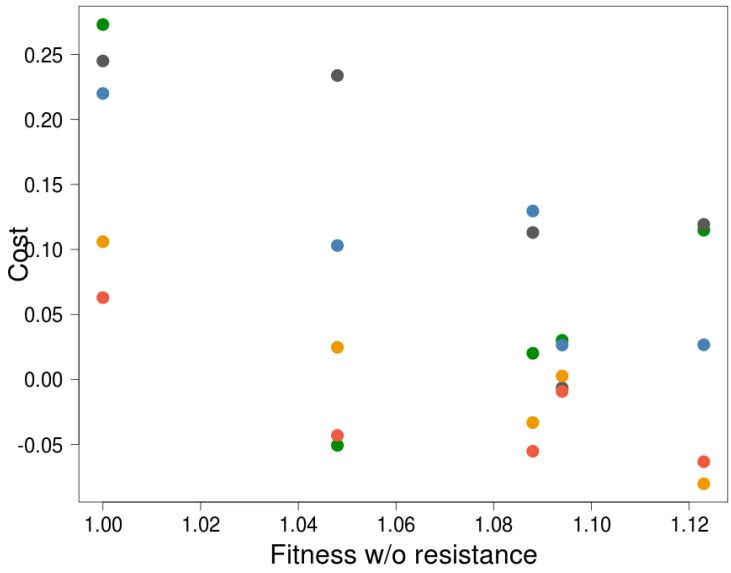
Introduction

One of the most remarkably consistent trends across experimental evolution studies is that genotypes with lower fitness are more evolvable than their high-fitness counterparts (for a meta-analysis see (COUCE AND TENAILLON 2015)). This implies that low-fitness genotypes may not be doomed to extinction and can rapidly catch up to, or even surpass high-fitness genotypes (WOODS *et al.* 2011). The negative relationship between evolvability and initial fitness is not because less-fit genotypes tend to fix more mutations during evolution (BARRICK *et al.* 2009; BARRICK *et al.* 2010; GIFFORD *et al.* 2011; KRYAZHIMSKIY *et al.* 2014). Rather, the benefit conferred by a given beneficial mutation tends to increase as the fitness of the genetic background it is in decreases (CHOU *et al.* 2011; KHAN *et al.* 2011; KRYAZHIMSKIY *et al.* 2014), and more beneficial mutations are accessible to low-fitness genotypes (FISHER 1930; SILANDER *et al.* 2007).

Results have been less clear, however, regarding how the fitness effects of random mutations change with the fitness of the genetic background into which they are inserted. Many studies have attempted to answer this question using mutation accumulation (MA) experiments where small populations are propagated such that mutations are fixed nearly randomly with respect to their effect on fitness (for a review see (HALLIGAN AND KEIGHTLEY 2009)). If the fitness effect of random deleterious mutations does not depend on the fitness of their genetic background, then there should be a linear relationship between the logarithm of fitness of a clone and the number of mutations it has acquired. Deviations from this linear relationship can be caused by systematic non-multiplicative interactions between mutations—called epistasis. There has been mixed evidence for epistasis in MA experiments and studies that have measured the fitness of two mutations when alone or in combination (reviewed in

(DE VISSER AND ELENA 2007)) with studies finding no epistasis (ELENA AND LENSKI 1997; HEILBRON *et al.* 2014), negative epistasis (multiple mutations are more deleterious than expected; e.g. (DICKINSON 2008)), and positive epistasis (multiple mutations are less deleterious than expected; e.g. (SANJUÁN *et al.* 2004; MAISNIER-PATIN *et al.* 2005)). Furthermore, many methodological criticisms have been levied against these studies.

The large difference in the form of epistasis between beneficial mutations or between deleterious mutations demonstrates that epistasis strongly depends on the nature of the mutations that are being combined (SZENDRO *et al.* 2013). Surprisingly, little research has been carried out on the effect of beneficial mutations in combination with deleterious mutations. Such work has important implications for the ease with which pathogens of different levels of adaptedness can acquire costly antibiotic resistance mutations. To our knowledge, only one study of this nature has been conducted thus far (DURÃO *et al.* 2015), in which the fitness effect of all possible pairwise combinations of 4 deleterious and 5 beneficial mutations were systematically quantified. In line with the literature on epistasis between beneficial mutations, they found that fitness benefit of the beneficial mutations were more beneficial in lower-fitness genotypes. While they did not explicitly test for this, their data therefore also show that the deleterious mutations are less costly in higher-fitness backgrounds (Supplementary Figure 1). These results lead to the tantalizing possibility that costly antibiotic resistance mutations would be less costly in well-adapted infectious bacteria, such as obligate pathogens, than species that are less well-adapted, such as opportunistic pathogens that are in the process of evolving to a novel host environment.



Supplementary Figure 1 Re-analyzed data from Durao *et al.* (DURÃO *et al.* 2015) showing that the cost of resistance decreases as the fitness of the genetic background into which it is inserted increases (partial F-test: P= 0.0004, F_1= 18.438; R^2= 0.593).

In the USA alone it is estimated that over 2 million people acquire serious infections that are resistant to at least one antibiotic that has been designed to treat that infection every year, resulting in more than 23,000 deaths (CENTERS FOR DISEASE CONTROL AND PREVENTION 2013). Mutations that confer resistance to antibiotics are typically costly in antibiotic-free environments (reviewed in (ANDERSSON AND HUGHES 2010)). As such, when a patient stops antibiotic treatment, antibiotic-sensitive bacteria may outcompete antibiotic-resistant bacteria, diminishing the threat of antibiotic resistance. Indeed, the cessation of antibiotic therapy in an individual or a reduction in the prescription of a particular antibiotic in a geographical region often (but not always) leads to a decrease in the frequency of resistance (ANDERSSON AND HUGHES 2010). However, many "cost-free" resistance mutations have been documented (see (MELNYK *et al.* 2015) for a meta-analysis) and the cost of plasmid-borne resistance genes tends to be minimal (VOGWILL AND MACLEAN 2015). The magnitude of the cost of resistance is therefore considered to be one of the key factors determining the spread and persistence of antibiotic resistance (ANDERSSON AND HUGHES 2010).

As such, we were interested in determining whether the cost of antibiotic resistance

mutations depends on the fitness of the genetic background in which they are found. To this end, we introduced three costly mutations that confer resistance into the antibiotic rifampicin into a rifampicin-sensitive ancestral strain of the opportunistic pathogen *Pseudomonas aeruginosa*, as well as into three strains from a brief evolution experiment that represent the first step in an adaptive walk.

Methods

Strains

In a previous study, we evolved 12 populations of the rifampicin-sensitive *P. aeruginosa* strain PAO1::mini-Tn7-pLAC-lux in M9KB medium at 30C with 225 RPM shaking for approximately 300 generations (Qı *et al.* in preparation). Three randomly-selected colonies from each endpoint population were sequenced using the HiSeq 2000 Sequencing System (Illumina, USA) and their whole-genome sequences were analyzed as described. Here, we selected three of these clones: two contained a single beneficial mutation, either in *dipA* or *morA*, and one contained beneficial mutations in both *dipA* and *lasR*. In addition, we selected 3 clones, each carrying a single rifampicin resistance mutation (D521G, H531R, or S536F) in the *rpoB* gene. Using fluctuation tests, we introduced these same rifampicin resistance mutations into all backgrounds carrying the aforementioned beneficial mutations, thereby generating all pairwise combinations of beneficial and resistance mutations.

Fluctuation assays

Fluctuation assays were performed as described in MacLean and Buckling (MACLEAN AND BUCKLING 2009). Briefly, we grew 5mL of each evolved strain in M9KB liquid medium and the wild-type overnight at 37°C with 225RPM shaking. Each culture was diluted 10^{-6} in M9KB and 90µL of diluted culture was added to each well of five 96-well microplates (~50 cells/well). Plates were incubated overnight at 37°C without shaking. Next, 5µL from each well was

spotted on M9KB agar plates containing 60µg/mL of rifampicin, which were then incubated at 37°C. After 48 hours, we picked 96 random colonies for *rpoB* sequencing and froze a sample of each in 50% glycerol at -80°C.

Sequencing of the rpoB gene

For each rifampicin-resistant clone we sequenced the region of the *rpoB* gene between nucleotides 1178 and 1864, which is known to carry rifampicin resistance mutations (MACLEAN AND BUCKLING 2009). To amplify DNA, colony PCR was performed using 12.5µL GoTaq HotStart Green Master Mix (Promega, USA), 1µL of forward primer (5'-GTTCTTCAGCGCCGAGCG-3'), 1µL of reverse primer (5'-GCGATGACGTGGTCGGC-3'), and 9.5µL nuclease-free water. PCR amplification was performed for 35 cycles using the following settings: initial denaturation (94°C for 5 minutes), denaturation (94°C for 30 seconds), anneal (60°C for 30 seconds), extension (72°C for 1 minute), final extension (72°C for 10 minutes). Purification of PCR products was performed using ExoSAP-IT (Affymetrix, USA) according to the manufacturer's instructions. BigDye sequencing of the purified PCR products was performed using the following reagents: 4.8µL PCR product, 3.2µL forward primer, 1.75µL BigDye Sequencing Buffer, and 0.25µL BigDye Ready Reaction Premix (Applied Biosystems, USA). The sequencing reaction was performed for 25 cycles using the following settings: initial denaturation (96°C for 1 minute), denaturation (96°C for 10 seconds), anneal (50°C for 5 seconds), extension (60°C for 4 minutes). BigDye sequencing reaction products were precipitated using ethanol/EDTA and were sequenced at the Sanger Sequencing Facility (Department of Zoology, University of Oxford).

Fitness assays

Competitive fitness assays were performed as described previously (HEILBRON *et al.* in preparation) with some minor modifications. Each strain was inoculated in 1mL M9KB and

incubated overnight at 37°C with 225RPM shaking. Cultures were then diluted 10-fold and grown to early exponential phase at 30°C for 2 hours with 225RPM shaking. Next, cultures were mixed 30:70 with the PAO1-GFP strain, diluted 200-fold, and incubated at 30°C for 20 hours. For each competition, the proportion of GFP-tagged cells was determined using a BD Accuri C6 flow cytometer (BD Biosciences, USA) before and after overnight co-incubation. Fitness was calculated as the number of doublings of the untagged strain relative to that of the GFP-tagged strain and all samples were standardized such that the relative competitive fitness of the rifampicin-sensitive ancestral strain (without and beneficial or resistance mutations) was assigned an arbitrary value of 1. Two replicate competitions were performed for each strain.

Statistical analysis

All statistical analyses were performed using R version 3.2.2 (R CORE TEAM 2015). Degrees of freedom are reported as subscripts of the test statistic and all tests are two-tailed, where applicable.

Results and Discussion

Experimental overview

In a previous study (QI *et al.* in preparation), we evolved 12 populations of *P. aeruginosa* PAO1 to rich medium for 300 generations. Using whole genome sequencing we identified clones carrying a single beneficial mutation in *dipA* or *morA* and a clone carrying beneficial mutations in both *lasR* and *dipA* (hereafter referred to as *lasR*/*dipA* and treated as a single beneficial locus as in (CHOU *et al.* 2011)). In order to investigate the fitness costs of rifampicin resistance mutations in low- and high-fitness genetic backgrounds, we used fluctuation assays to evolve 3 rifampicin resistance mutations in the *rpoB* gene (D521G, H531R, or S536F) in each strain carrying beneficial mutation(s), as well as in the rifampicin-sensitive ancestral strain. Given that these strains only acquired 1-2 beneficial mutations in 300

generations, it is highly unlikely that they acquired more than one mutation during the fluctuation assays (~23 generations), making this technique preferable to allelic replacement. The competitive fitness of each strain was measured via flow cytometry in order to determine whether the cost of rifampicin resistance was dependent on genetic background.

The individual fitness effects of resistance and beneficial mutations

The 3 rifampicin resistance mutations that we studied are all non-synonymous mutations in the beta subunit of RNA polymerase (encoded by the *rpoB* gene), and will hereafter be referred to by their position and amino acid substitution: D521G, H531R, and S536F. All 3 rifampicin resistance mutations conferred a significant fitness cost (Figure 1; one-sample *t*-test: $P < 0.05$), partially due to compromised transcriptional efficiency of the RNA polymerase holoenzyme (QI *et al.* 2014). The magnitude of these costs was similar to that found for these same mutations in other studies (MELNYK *et al.* 2015; VOGWILL *et al.* in preparation).

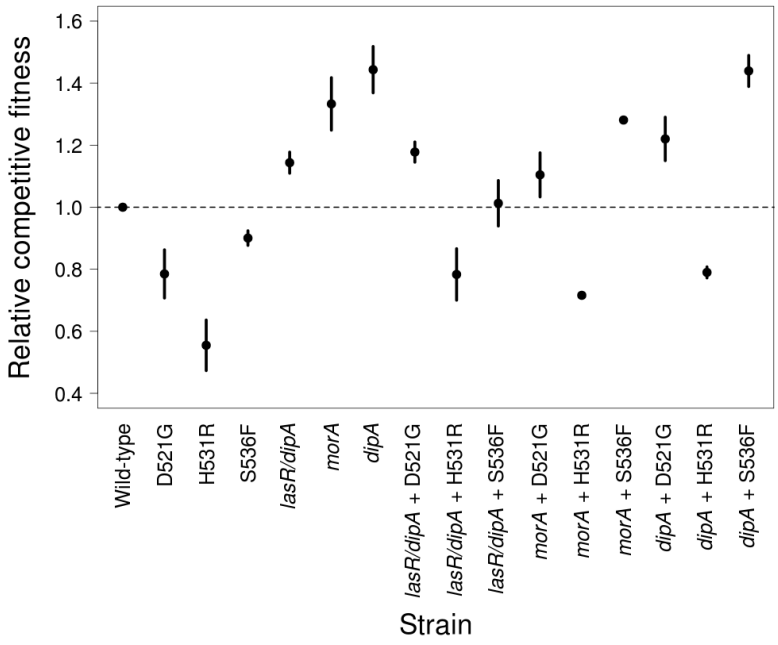


Figure 1 Fitness (± SE) relative to the wild-type for 3 costly rifampicin resistance mutations, 3 beneficial mutations, and all 9 possible pairwise combinations of resistance and beneficial mutations. The dashed line represents the wild-type fitness.

On the other hand, the 3 beneficial mutations were each located in different genes (*morA, dipA*, and *lasR/dipA*) and all conferred a significant fitness benefit (one-sample *t*-test: $P< 0.05$) (Figure 1). Both *morA* and *dipA* are involved in regulating cellular levels of cyclic di-GMP (c-di-GMP), a secondary messenger that coordinates many phenotypes, such as motility, virulence factor production, and biofilm formation. *morA* mutations have been shown to confer a fitness benefit in a laboratory environment similar to that used in our selection experiment, possibly by decreasing motility (HEILBRON *et al.* in preparation). *lasR* mutations such as the P74L mutation we observed in our study lead to defects in quorum-sensing and are commonly fixed during adaptation to novel environments (HEURLIER *et al.* 2005; D'ARGENIO *et al.* 2007). It is noteworthy that the strain carrying both a *lasR* and a *dipA* mutation was less fit than the strain carrying a single *dipA* mutation. There are two possible reasons for this discrepancy. First, since the two *dipA* mutations are different (frameshifts at different positions), they may have different fitness effects. Second, the *lasR* mutation may have arisen first and may have epistatically attenuated the fitness benefit of the *dipA* mutation. Although this result was surprising, it enabled us to compare the cost of rifampicin resistance in genetic backgrounds with a wider range of fitness levels.

The combined fitness effects of resistance and beneficial mutations

We found no significant relationship between the cost of resistance and the fitness of the genetic background into which a resistance mutation was introduced (Figure 2A; partial F-test: $P= 0.853$, $F_1= 0.0366$). However, we found significant positive epistasis—the fitness of double-mutants carrying both a resistance mutation and a beneficial mutations had higher fitness than expected if these mutations had independent effects on fitness (Figure 2B; one-sample *t*-test: $P= 0.036$, $t_8= 2.516$). Specifically, the average cost of resistance mutations in the wild-type was 25.3%, but the average cost in backgrounds that contained a beneficial mutation was only 18.7%. Thus, although the cost of resistance was not affected by the fitness of the

genetic background, the cost of resistance differed in the wild-type versus in clones that had taken one beneficial step in an adaptive walk. These results are similar to those from recent work on combinations of beneficial and deleterious antibiotic resistance mutations in *E. coli* (DURÃO *et al.* 2015).

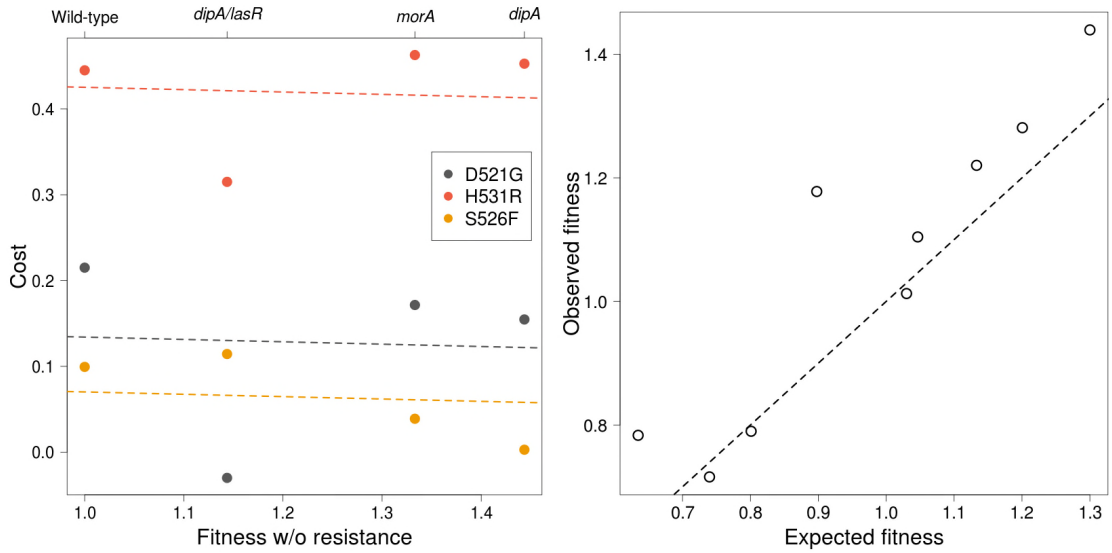


Figure 2 (A) The cost of 3 rifampicin resistance mutations in genetic backgrounds with various fitness levels. The dashed lines represent the fit of a model where cost is explained by background fitness (numerical) and resistance mutation (categorical), but no interaction between them. This model is not significantly better than a model that is identical, but does not include background fitness (partial F-test: P= 0.853). **(B)** Epistasis between beneficial and resistance mutations. Expected fitness is the product of the fitness values of the two single mutants. The dashed line represents the 1:1 line where observed and expected fitness are equal. There is significant positive epistasis (points falling above the 1:1 line, t-test: P= 0.036).

The positive epistasis that we observed not only means that rifampicin resistance mutations are less costly in backgrounds containing beneficial mutations—by necessity it also means that beneficial mutations confer a greater fitness advantage in rifampicin-resistant backgrounds. This may explain why a study by Barrick *et al.* (BARRICK *et al.* 2010) found that decreased fitness due to rifampicin resistance mutations in *Escherichia coli* led to increased evolvability. Several other studies that have engineered different combinations of beneficial mutations have found similar results (CHOU *et al.* 2011; KHAN *et al.* 2011; KRYAZHIMSKIY *et al.*

2014). However, these studies have found “diminishing returns epistasis” whereby the fitness gain engendered by a beneficial mutation increases as the fitness of the host genotype into which it is engineered decreases. In our study, beneficial mutations conferred a greater benefit in resistant backgrounds compared with the wild-type, but there was no relationship between the fitness gain conferred by a beneficial mutation and the fitness of the background into which it was added.

Why was there a difference in the cost of resistance between the wild-type and beneficial backgrounds, but no overall relationship between cost and the adaptedness of the genetic background? One possibility is that the beneficial mutations increased fitness via a common phenotypic mechanism. For example, previous work has shown that the cost of rifampicin resistance decreased with reduced demand for RNA polymerase activity (HALL *et al.* 2011). Since all beneficial mutations affected genes involved in regulating the transcription of a large number of genes, it is possible that the beneficial mutations led to overall decreases in gene expression, thereby offsetting the cost of transcriptional inefficiency. Indeed, the expression of 6-10% of the *P. aeruginosa* genome is regulated by quorum-sensing (SCHUSTER *et al.* 2003), which is in turn regulated by *lasR* (SANDOZ *et al.* 2007). Further experiments are necessary to test this hypothesis, possibly by comparing the transcriptional profiles of these strains.

Conclusion

We have found that the cost of a deleterious mutation does not depend on the fitness of the background into which that mutation is introduced. However, we have shown that it is possible for the cost of resistance to an antibiotic to decrease as a result of adaptive evolution. Further work will need to be done in order to determine the circumstances under which this will be the case. More broadly, more experiments are needed to determine whether pathogens that are well-adapted to their hosts are more capable of acquiring antibiotic

resistance than those that are not.

General Discussion

Since this thesis is comprised of a collection of manuscripts, each of the preceding data chapters contains its own Discussion section. The purpose of this General Discussion is to highlight some of the key findings of my work, discuss possible avenues for future research, and provide some concluding thoughts on how the data chapters relate to one another.

Key findings

Chapter 1

Natural selection during mutation accumulation (MA): I found little evidence that natural selection biased the types of mutations that were fixed during MA, with the exception of frameshift mutations. Previous studies that likewise combined MA experiments with whole genome sequencing have also failed to detect a molecular signature of natural selection during MA (including frameshifts), but the number of mutations examined in these studies was relatively small. I was able to circumvent this problem by using a hypermutator strain, providing us with much greater power to detect signatures of natural selection. This result was surprising to some extent because microbial MA experiments require several generations of growth during which natural selection is possible in order to obtain visible colonies. Indeed several other microbial MA studies have also found evidence that beneficial mutations were fixed during the experiment (JOSEPH AND HALL 2004; PERFEITO *et al.* 2007; DICKINSON 2008; TRINDADE *et al.* 2010; STEVENS AND SEBERT 2011).

The distribution of fitness effects (DFE) of spontaneous mutations: I estimated that 42.3% of the decrease in fitness in our experiment was driven by 0.5% of the mutations. Much previous work has modelled the DFE as a displaced gamma distribution (SHAW *et al.* 2002) because it presents several attractive qualities: a minority of exponentially distributed beneficial mutations (ORR 2002), a mode that is nearly neutral (KIMURA 1983), and a left-hand tail that

allows for rare mutations of large deleterious effect. In fact, the assumptions of Fisher's geometric model of adaptation lead to a displaced gamma DFE as an emergent property (FISHER 1930; MARTIN AND LENORMAND 2006). My work and the work of others, however, have suggested that this may be an oversimplification and that the true DFE of spontaneous mutations may be multimodal (DAVIES *et al.* 1999; HALLIGAN AND KEIGHTLEY 2009).

Mutation spectrum: In our Δ*mutS* hypermutator strain: 99.5% of base substitutions were transitions, 17.4% of mutations were short indels, and 95.3% of these indels were in homopolymeric tracts. These results have helped to explain some of the findings of Hammerschmidt and colleagues (HAMMERSCHMIDT *et al.* 2014). They found that a mutation in *mutS* was critical for enabling rapid switching between two phenotypes by allowing insertions and deletions in a homopolymeric tract of guanines in the active site of the *wspR* gene.

Chapter 2

Ruggedness of the adaptive landscape may increase with distance from a fitness peak: I found several lines of evidence that suggested that recovery populations from low-fitness founders experienced a more rugged fitness landscape than those founded from high-fitness founders. First, populations founded by the MA line with the lowest initial fitness displayed impaired evolvability. Second, populations founded by the two low-fitness MA lines (but not those from the two high-fitness MA lines) exhibited a significantly greater degree of within-line convergence compared to between-line convergence (see Chapter 2 for a more comprehensive description). Finally, mutations in *morA* were significantly enriched in recovery clones from a low-fitness MA line that had previously acquired a *morA* mutation during MA, suggesting that this MA mutation potentiated a different evolutionary trajectory in these clones. The idea that ruggedness increases with distance to a fitness peak has recently been proposed (NAHUM *et al.* 2015) to explain a discrepancy in the results from a study that found abundant ruggedness in low-fitness *E. coli* (NAHUM *et al.* 2015) and another study that found

little ruggedness in high-fitness *S. cerevisiae* (KRYAZHIMSKIY *et al.* 2012).

Intragenic compensation contributed little to adaptation: Genes that were mutated during MA were not significantly more likely to acquire another mutation during the recovery phase. Although this result was surprising since 92% of compensatory mutations are estimated to be intragenic (POON *et al.* 2005), a similar study in *C. elegans* has also found similar results (DENVER *et al.* 2010). Instead, genes that exhibited a strong signature of natural selection were involved in motility and the production of virulence factors and siderophores—phenotypes which may be important for growth in a host or in the environment, but are not essential for growth in shaking broth culture under laboratory conditions. This indicates that compensatory mutations may have been outcompeted by "laboratory adaptation mutations", a phenomenon that has previously been reported in this system (QI *et al.* in preparation).

morA and motility: I found that recovery clones bearing *morA* mutations had decreased motility. In contrast, a previous paper reported that while *morA* played a role in motility in *Pseudomonas putida*, it had no such effect in *P. aeruginosa* (CHOY *et al.* 2004). Although both studies were conducted using the *P. aeruginosa* PAO1 strain, there are substantial genomic differences between PAO1 strains, including a 2.2Mb inversion (KLOCKGETHER *et al.* 2010). I have therefore shown that the role of *morA* in motility depends upon its genetic background, contributing to our understanding of the function of the *morA* gene in *P. aeruginosa*.

Chapter 3

It is possible for adaptation to decrease the cost of resistance: On average, the fitness cost of rifampicin resistance was 25.3% in the wild-type background and 18.7% in backgrounds containing a beneficial mutation. This did not manifest in a relationship between the cost of resistance and background fitness, however. Previous work (DURÃO *et al.* 2015) did find that cost significantly decreased with increasing background fitness, but did not specifically report this result in their paper. Further work is necessary to disentangle these two results and

determine the conditions under which adaptation alters the cost of resistance.

Future directions

Chapter 1

Mutation accumulation experiments have long been an important source of data on the rate and effect of spontaneous mutations (BATEMAN 1959; MUKAI 1964), and advances in next-generation DNA sequencing technology have made it possible to study these mutations in greater detail than ever before. We now have good estimates of the spontaneous mutation rate in many organisms (LYNCH 2010), but an accurate description of their DFE is still lacking (HALLIGAN AND KEIGHTLEY 2009). The DFE is typically determined indirectly using a maximum likelihood method to estimate the parameters of a pre-specified distribution based on the change in mean fitness and the change in variance in fitness across MA lines over time (KEIGHTLEY 1994; GARCÍA-DORADO 1997; SHAW *et al.* 2002).

In order to directly determine the DFE I would like to see the following experiment conducted. A large number of MA lines would be propagated for a short amount of time such that many endpoint clones are expected to have fixed a single mutation. These clones would be sequenced and those that did not contain exactly one mutation would be discarded. Measuring the fitness of these clones would provide a direct measure of the DFE. To directly measure how the effect of these mutations depends on the fitness of their genetic background, these same mutations could then be engineered into other strains with various fitness levels, such as a sequence of related clones from a line from Richard Lenski's long-term evolution experiment (LENSKI AND TRAVISANO 1994).

Chapter 2

In Chapter 2 I showed that there was little evidence of intragenic compensation.

Rather, genes that were repeatedly mutated were involved in the production of costly secreted proteins or activities, such as the synthesis of virulence factors and siderophores. Given that these genes were mutated during the recovery phase in populations from different MA line and from the controls, these mutations most likely represent adaptation to laboratory conditions, not compensation for a specific mutation acquired during MA. Although laboratory adaptation was much stronger than compensation in this experiment, the relative influence of laboratory adaptation should decrease as the strain under consideration becomes better adapted to its environment.

I would therefore be interested to investigate how the results of this experiment would change if I repeated the experiment with a clone that had first been adapted to the experimental conditions for several thousand generations. In this highly-adapted clone, the fitness gain conferred by new beneficial mutations would be smaller than the average fitness cost of spontaneous mutations if this clone were to be subjected to MA. It would be interesting to determine the extent to which recovery clones from different MA lines would fix mutations in the same genes in this new experiment. Since the positive effect of a beneficial mutation tends to increase in a low-fitness background (CHOU *et al.* 2011; KHAN *et al.* 2011; KRYAZHIMSKIY *et al.* 2014), laboratory adaptations may still be able to fix in recovery clones across different MA lines. If compensatory mutations did play a larger role in this new experiment, it would be interesting to see whether compensatory mutations tend to occur in the genes that led to the greatest loss of fitness when mutated during MA. In MA lines where the cost of all spontaneous mutations is small, it would be interesting to determine whether any recovery mutations confer a fitness benefit that is greater than the cost of any single spontaneous mutation. This may suggest that a single recovery mutation is compensating for multiple MA mutations simultaneously.

Chapter 3

Two recent meta-analyses have attempted to determine the factors that influence the cost of antibiotic resistance (MELNYK *et al.* 2015; VOGWILL AND MACLEAN 2015). One potential factor that has received little attention, however, is the fitness of the strain being subjected to antibiotic pressure. On the other hand, there is ample evidence that the fitness of an organism affects the fitness gain conferred by a beneficial mutation (CHOU *et al.* 2011; KHAN *et al.* 2011; KRYAZHIMSKIY *et al.* 2014).

My work in Chapter 3 and the work of others (DURÃO *et al.* 2015) have begun to explore whether the cost of resistance is affected by background fitness, but the results of these two studies are somewhat discordant. In order to further examine this potential relationship, I would like to perform the following experiment. A large number of fluctuation tests would be conducted for several antibiotics in strains with various fitness levels, such as a sequence of related clones from a line from Richard Lenski's long-term evolution experiment (LENSKI AND TRAVISANO 1994). Targeted or whole genome sequencing would be used to identify clones containing a single resistance mutation. With a sufficiently large sample size, the same mutation would appear in many different backgrounds and the relationship between the cost of resistance and background fitness could be elucidated for each such mutation and each antibiotic. Allelic replacement could be used to engineer combinations that were not recovered from the fluctuation assays. Taken together, these data could be used to determine if there are predictable conditions under which the cost of resistance will be affected by background fitness.

Conclusion

Through the work in hypermutator *P. aeruginosa* that I have presented in this thesis, I have contributed to our understanding of how the fitness of a genetic background interacts with the fitness effect of incoming mutations. In Chapter 1, I performed a mutation

accumulation (MA) experiment and found no evidence that the fitness effect of spontaneous mutations changed systematically as fitness decayed. Directly following on from this work, I selected four MA line endpoints in Chapter 2 and evolved them in large populations to study how adaptive evolution differed in genotypes separated by a large number of spontaneous mutations. My results supported the well-known trend that adaptive evolution due to the acquisition of beneficial mutations depends on the fitness of the genetic background. However, I also found that low-fitness backgrounds experienced a more rugged fitness landscape, possibly resulting in populations occupying lesser peaks. In Chapter 3 I further explored how the fitness of a genetic background can influence evolution in the context of the acquisition of antibiotic resistance. I found that while there is no overarching relationship between background fitness and the cost of resistance, acquiring 1-2 beneficial mutations consistently decreased the cost of resistance relative to the wild-type strain in this system. Taken together, my work has highlighted how the fitness of a strain can influence the outcome of evolution depending on the nature of the mutations under consideration.

Appendix: The genomic basis of adaptation to the fitness cost of rifampicin resistance in *Pseudomonas aeruginosa*

Qin Qi[1, 2], Macarena Toll-Riera[1, 3], Karl Heilbron[1], Gail M. Preston[4], R. Craig MacLean[1]

1. Department of Zoology, University of Oxford, United Kingdom
2. Current affiliation: Institute of Science and Technology Austria, Klosterneuburg, Austria
3. Current affiliation: Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Switzerland
4. Department of Plant Sciences, University of Oxford, United Kingdom

Corresponding authors: Qin Qi (qin.qi06@alumni.imperial.ac.uk) or R. Craig MacLean (craig.maclean@zoo.ox.ac.uk)

Abstract

Antibiotic resistance carries a fitness cost that must be overcome in order for resistance to persist over the long term. Compensatory mutations that recover the functional defects associated with resistance mutations have been argued to play a key role in overcoming the cost of resistance. However, compensatory mutations are thought to be rare relative to generally beneficial mutations that increase fitness irrespective of antibiotic resistance. Given this asymmetry, theory predicts that populations should adapt by compensatory mutations when the cost of resistance is large, while generally beneficial mutations should drive adaptation when the cost of resistance is small. We tested this prediction by determining the genomic mechanisms underpinning adaptation to antibiotic-free conditions in experimental populations of the pathogenic bacterium *Pseudomonas aeruginosa*. Whole-genome sequencing of evolved clones revealed that populations founded by high cost

rifampicin-resistant mutants adapted via compensatory mutations in three genes of the RNA polymerase core enzyme, whereas populations founded by low-cost mutants adapted by generally beneficial mutations, predominantly in the quorum sensing transcriptional regulator gene *lasR*. Our study suggests that compensatory evolution was rare, because generally beneficial mutations were very common relative to compensatory mutations, and often resulted in large gains in fitness. Our study challenges the importance of compensatory evolution in the maintenance of resistance and highlights the need to understand how selection at other sites in the genome influences the dynamics of resistance alleles in clinical settings.

Introduction

The evolution of antibiotic resistance in pathogenic bacteria is typically accompanied by fitness costs that are expressed in terms of reduced growth rate, competitive ability and virulence (ANDERSSON 2006; ANDERSSON AND HUGHES 2010; MACLEAN *et al.* 2010a). Fitness costs generate selection against resistance when pathogen populations encounter antibiotic-free environments, as occurs during transmission between hosts or when antibiotic use is discontinued. Since exposure to high doses of antibiotic is transient, resistance will only be maintained in the long term if resistant populations can evolve adaptations that offset the cost of resistance. Therefore, understanding the mechanisms that allow resistant bacterial populations to evolve increased fitness is of fundamental importance to understanding the long-term maintenance of resistance in pathogenic bacteria.

One possible mechanism through which bacteria can overcome the cost of resistance is by acquiring compensatory mutations that reduce or eliminate the fitness costs associated with resistance alleles by recovering the functional defects associated with resistance mutations (REYNOLDS 2000; NAGAEV *et al.* 2001; MAISNIER-PATIN AND ANDERSSON 2004; ZORZET *et al.* 2010; BRANDIS AND HUGHES 2013; QI *et al.* 2014). Alternatively, it is possible for antibiotic

resistant populations to overcome the fitness cost of resistance by acquiring generally beneficial mutations that increase fitness without offsetting the cost of resistance *per se*. Unlike compensatory mutations, generally beneficial mutations contribute to an increase in fitness regardless of the genetic background in which they arise (ZHANG AND WATSON 2009; ESTES *et al.* 2011). In this scenario, resistant populations overcome the cost of resistance by simply adapting to general environmental conditions, such as nutrient availability and the presence of stressors. The dominant view that has emerged from studies of the long-term evolution of antibiotic resistant populations is that adaptation to the cost of resistance is driven by compensatory mutations (ANDERSSON AND LEVIN 1999; ANDERSSON 2006; MACLEAN *et al.* 2010a; SCHULZ ZUR WIESCH *et al.* 2010; HUGHES AND ANDERSSON 2015). Compensatory mutations have been identified in clinical pathogen populations, especially *M. tuberculosis* (COMAS *et al.* 2012; DE VOS *et al.* 2013; PERDIGÃO *et al.* 2014), and compensatory adaptation has emerged as a central explanation for the long-term maintenance of resistance in pathogen populations (ANDERSSON AND HUGHES 2010).

Although compensatory mutations have been identified in a wide range of systems, current discussions of evolution in resistant populations tend to overlook the fact that resistant populations can also evolve by generally beneficial mutations that increase fitness irrespective of antibiotic resistance. It has been estimated that there are on average 12 possible compensatory mutations per deleterious mutation in bacteria (POON *et al.* 2005), and the rate of compensatory mutation should therefore be on the order of 12 sites/genome x 9 x 10^{-11} mutations per site per generation under laboratory conditions (WIELGOSS *et al.* 2011), which corresponds to ≈1 x 10^{-9} per genome per generation. This is quite a crude approximation, but the important point is that estimates of the overall genomic rate of beneficial mutation are usually much higher than this estimate. For example, generally beneficial mutations have been estimated to occur at a rate between 1 x 10^{-8} and 1 x 10^{-5} per genome per generation under laboratory conditions, which is 10 to 10 000 times higher than

our crude estimate of the rate of compensatory mutation (IMHOF AND SCHLOTTERER 2001b; BARRETT *et al.* 2006; PERFEITO *et al.* 2007). This asymmetry between the rate of compensatory mutation and the overall rate of beneficial mutations suggests that, all else being equal, adaptation in antibiotic resistant populations should be driven primarily by generally beneficial mutations. However, adaptation in asexual populations is driven by a small minority of mutations with relatively large benefits that manage to overcome stochastic drift and competition from rival beneficial mutations due to clonal interference (ROZEN *et al.* 2002; BARRETT *et al.* 2006; PERFEITO *et al.* 2007; BELL 2008). Compensatory mutations should therefore make a disproportionately large contribution to adaptation when they are associated with large fitness benefits relative to generally beneficial mutations. Because compensatory mutations recover the functional defects associated with resistance mutations (REYNOLDS 2000; BRANDIS *et al.* 2012; BRANDIS AND HUGHES 2013; QI *et al.* 2014), compensatory mutations should be associated with large fitness benefits in populations carrying very costly resistance mutations and small fitness benefits in populations carrying resistance mutations that incur a small cost. This simple population genetic framework predicts that the likelihood of adaptation by compensatory mutations should increase with the cost of resistance (SZAMECZ *et al.* 2014). Crucially, many studies of evolution in antibiotic resistant populations have largely focussed on adaptation in populations that carry very costly resistance mutations (SCHRAG AND PERROT 1996; BJÖRKMAN *et al.* 2000; NAGAEV *et al.* 2001; MAISNIER-PATIN *et al.* 2002; KUGELBERG *et al.* 2005; NILSSON *et al.* 2006). If the predictions of our hypothesis are correct, the role played by compensatory mutations in eliminating the fitness cost of antibiotic resistance may be smaller than currently thought.

To test the hypothesis that fitness costs influence the molecular mechanisms of adaptation to the cost of antibiotic resistance, we allowed populations of 8 isogenic rifampicin-resistant clones of *P. aeruginosa* that carry different fitness costs, as well as their rifampicin-sensitive ancestor, to evolve for 300 generations in an antibiotic-free medium. Extensive

whole-genome sequencing of evolved clones from antibiotic resistant and antibiotic-sensitive control populations was used to systematically identify compensatory mutations and test the hypothesis that high fitness costs promote evolution by compensatory adaptation.

Results and Discussion

Evolved rifampicin-resistant mutants recover from the fitness cost of resistance

To study adaptation to the cost of rifampicin resistance, we allowed 3 independently propagated populations of 8 different rifampicin-resistant *rpoB* mutants that carried different fitness costs to evolve in a rifampicin-free culture medium for 300 generations. Fitness varied considerably between the rifampicin-resistant mutants that were used to initiate the selection experiment (Figure 1: one-way ANOVA, $F_{7,16} = 4.66$, $P = 0.0052$). Fitness increased over the course of the selection experiment (paired *t*-test, $t_7 = 5.47$, $P = 0.0009$), and the fitness of the evolved populations was very similar to that of the rifampicin-sensitive control strain that the resistant mutants were evolved from. These results clearly demonstrate that selection in the absence of antibiotics can rapidly eliminate the substantial fitness cost that is associated with rifampicin resistance for a range of *rpoB* mutations.

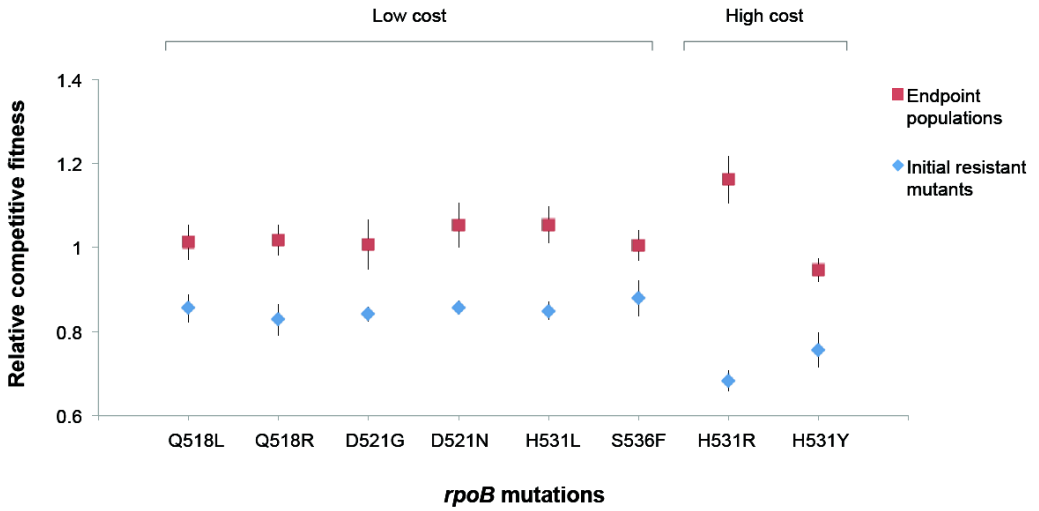


Figure 1 Adaptation in endpoint populations evolved from rifampicin-resistant mutants. This figure shows competitive fitness (mean ± s.e; n = 3) of the 8 *rpoB* mutants used to found the selection experiments (blue diamonds) and the average fitness of 3 endpoint populations (red squares) evolved from each *rpoB* mutant. The relative fitness of all endpoint populations converged towards that of the rifampicin-sensitive ancestral strain (standardised to a value of 1). In all cases, the average relative fitness of the endpoint populations showed a significant increase compared to that of the initial *rpoB* mutant (two-sample *t*-test, $P < 0.05$).

Testing for compensatory adaptation using whole-genome sequencing

To determine the genetic basis of adaptation in antibiotic resistant populations, we performed whole-genome sequencing on 3 randomly selected colonies isolated from each evolved endpoint population (Table 1: 8 resistant mutants * 3 populations/mutant * 3 clones/population = 72 evolved clones). As a control experiment, we also sequenced the genomes of evolved clones from rifampicin-sensitive control populations that were allowed to adapt to antibiotic-free culture medium for the same number of generations (Table 2: 12 populations * 3 clones/population = 36 evolved clones). Non-synonymous SNPs (single-nucleotide polymorphisms) and short indel (insertion and deletion) mutations of <1 kb in protein-coding regions were the most common forms of mutations that we identified. Silent and intergenic mutations were found at low frequencies, as were large deletions and duplications spanning more than 1 gene.

Table 1. Genome-wide mutations in sequenced endpoint clones evolved from rifampicin-resistant *rpoB* mutants

rpoB mutation	Genes	Endpoint clones								
		Population A			Population B			Population C		
		1	2	3	1	2	3	1	2	3
Q518L	Intergenic, upstream of PA0040								+ 4 bp (40542)	
	PA0829				I172T					
	lasR (PA1430)	Q98*			Δ 66 bp (1558210)	W88*	Δ 66 bp (1558210)	GAAAA→GAAA (1558214)		S129I
	bkdA1 (PA2247)				Δ 213 bp (2473338)					
	rpoA (PA4238)								S49F	
Q518R	*lasR* (PA1430)				K16* A231V	A231V	V208E	R180W		A231V
	wzz (PA3160)							T50A		
	arnT (PA3556)				Silent					
	rpoA (PA4238)								D198V	
	rpoC (PA4269)						D410G			G367S
	rpoB (PA4270)	S528D			S528D					
D521G	*lasR* (PA1430)	S106*			Δ 56 bp (1558201)			T75P	L84M	T75P
	tufB (PA4277)				F6I					
	cupE6 (PA4653)						F124L			
D521N	*lasR* (PA1430)	ACT→AC TCT (1558403)					A227V	Δ 147 bp (1558339)	Δ 358 bp (1558129)	
	lasR and several adjacent genes				Δ 3873 bp (1556100)	Δ 12457 bp (1550983)				
	Several genes related to biofilm formation (PA2228 -51)		Δ 29106 bp (2450096)							
H531L	*fha1* (PA0081)						+ 6 bp (98901)			
	lasR (PA1430)		Δ 217 bp (1558109)	GA→GAA (1558450)						Δ 13 bp (1558370)
	lasR and several adjacent genes	Δ 3139 bp (1556268)								
	Intergenic between PA1519 and PA1520	SNP								
	cupE6 (PA4653)				F124L			S185R		
H531R	*lasR* (PA1430)	Δ 217 bp (1558172)								
	Several genes (PA2225-28)				Duplication: 2683 bp					
	Several genes (PA2339-86)				Δ 55682 bp (2583632)		Δ 55682 bp (2583632)			
	rpoB (PA4270)	H531C			H531C			Reversion		
H531Y	PA4673.16 #									R311H
	PA1829			D95T						
	PA1944						S336A			
	Intergenic, downstream of *pvcD* (PA2257) and *ptxR* (PA2258)									GT →AC (2486285)
	gacA (PA2586)	Q16*								
	Intergenic, upstream of *fptA* (PA4221)					G →A (4726833)				
	rpoB (PA4270)			N573S	N573S			H531C		
S536F	*gacS* (PA0928)			Q402*						
	lasR (PA1430)	Δ 11 bp (1558210)	Δ 20 bp (1558151)	CTT→CT (1558320)		T → TA (155844)	GAAAA → GAAA (1558573)			
	lasR and PA1429							Δ 2058 bp (1556368)		
	PA2067					Silent				
	Intergenic, upstream of *pvdG* (PA2425) and *pvdS* (PA2426)					G →T (2722079)				
	selA (PA4808)					Silent				

Table cells are shaded using different colours to represent the various types of mutations: nonsense mutation (red), nonsynonymous SNP (blue), synonymous SNP (green), indel (insertion and deletion) mutation (yellow), duplication (purple), reversion mutation (black). Specific mutations are described in text within each cell. For nonsense mutations, the asterisk sign (*) represents the stop codon. The numbers in parentheses represent the starting position of nucleotides in the *P. aeruginosa* PAO1 reference genome (NC_002516.2) at which the mutations were found. # PA4673.16 is a putative protein kinase gene of the RGP42 phage (reference genome: GQ141978.1) and was found in the genome of the PAO1 strain used in this study.

Table 2. Genome-wide mutations in sequenced endpoint clones evolved from the rifampicin-sensitive ancestral strain

Population	Endpoint clone	Genes											
		lecCDABE	Intergenic between PA0172 and PA0173	*lasR* (PA1430) ##	Intergenic between PA1436 and PA1437	Intergenic between PA2228 and PA2229	PA2718	*wspF* (PA3703)	*wspA* (PA3708)	PA3718	*groEL* (PA4385)	*morA* (PA46011)	*dipA* (PA5017)
A	1												Δ 63 bp (5641321)
	2					Δ 122 bp (2451377)							
	3												
B	1					Δ 122 bp (2451365)						R1019H	
	2												
	3												D507A
C	1			M1I (loss of start codon)									R533H
	2							CA → C (4145326)					
	3												
D	1												AC → A (5641110)
	2							TAA → TAAA (4145159)					
	3		G→T (196819)										
E	1											G1343S	
	2							GAAAAA →GAAAA (4145176)					
	3	Δ *lecCDABE*											Δ 10 bp (5641144)
F	1			Δ 248 bp (1557881)									Δ 120 bp (5641151)
	2							CT → C (4145380)					
	3			Δ 248 bp (1557881)									Δ 120 bp (5641151)
G	1								A418V				
	2			E196D									+ 13 bp (5642437)
	3												L518P
H	1			P74L									TGG → TG (5642258)
	2								V474L				
	3												
I	1				A→G (1567169)				A418V				
	2		G→T (196819)							F390			
	3								A418V				
J	1												Δ 10 bp (5641144)
	2												
	3												Δ 18 bp (5642795)
K	1										R322C		Δ 10 bp (5641144)
	2												
	3							S252F					
L	1							G61D					
	2												
	3						Silent						

Table cells are shaded using different colours to represent the various types of mutations: nonsynonymous SNP (blue), synonymous SNP (green), indel (insertion and deletion) mutation (yellow). Specific mutations are described in text within each cell. For nonsense mutations, the asterisk sign (*) represents the stop codon. The numbers in parentheses represent the starting positions of the nucleotide in the *P. aeruginosa* PAO1 reference genome (NC_002516.2) at which the mutations were found. ## The *lasR* mutations listed in this table include a deletion mutation in the intergenic region 41 bp upstream from the *lasR* start codon, which resulted in the loss of the *lasR* promoter region.

Identifying compensatory mutations

Since compensatory mutations interact epistatically with resistance mutations to increase fitness (SCHRAG *et al.* 1997; BJÖRKMAN *et al.* 2000; BRANDIS *et al.* 2012), we expected compensatory mutations to be found exclusively in evolved resistant populations, but not in rifampicin-sensitive control populations that were evolved for the same number of generations. By comparing the mutations found in a large sample of resistant and sensitive populations, it should be possible to identify compensatory mutations in a comprehensive and unbiased manner. Because our evolved clones are likely to carry both generally beneficial mutations that spread to high frequency as a result of positive selection, as well as neutral or mildly deleterious mutations, we restricted this analysis to genes that were mutated in more than one clone to focus on generally beneficial mutations. Such parallel evolution is common in experimental evolution and natural pathogen populations, and it provides a simple hallmark of genes that are under strong positive selection (LIEBERMAN *et al.* 2011; TENAILLON *et al.* 2012; FARHAT *et al.* 2013; STERN 2013). Our results broadly indicate that such parallel evolution was very common in both the evolved rifampicin-resistant and control populations. Amongst the resistant mutant populations, 5 genes (in blue, Figure 2) contained 76% of all mutations that were detected in the sequenced endpoint clones, while 6 genes (in red) covered 90% of all mutations found in the evolved control populations.

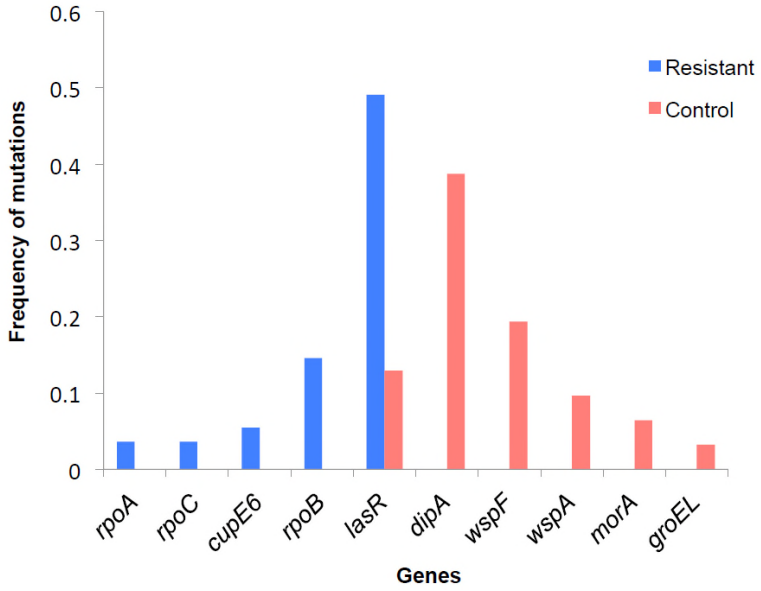


Figure 2 Most frequently mutated genes. This graph shows the frequency distribution of mutation in genes that were mutated in at least two independently evolved clones. A high degree of parallel evolution was observed in both the rifampicin resistant and control populations. A distinct difference in the spectrum of genes that were most frequently mutated can also be observed between the rifampicin-resistant and control groups.

Previous works have shown that second-site mutations in the subunits of the RNA polymerase core enzyme (*rpoA, rpoB* and *rpoC*) can compensate for the cost of rifampicin resistance (BRANDIS *et al.* 2012; BRANDIS AND HUGHES 2013; DE VOS *et al.* 2013; QI *et al.* 2014). Consistent with this, we found 9 independently evolved point mutations in these RNA polymerase genes amongst the resistant populations, but no RNA polymerase mutations were detected in the evolved control populations. Interestingly, one of the *rpoB* mutations represents a reversion mutation that swept to fixation in a population that was initiated with the H531R mutation, resulting in an evolutionary reversal of antibiotic resistance. Several lines of evidence suggest that the remaining RNA polymerase mutations are compensatory mutations rather than generally beneficial mutations. First, we demonstrated previously that the second-site intragenic mutations in *rpoB* observed in this study can directly recover the reductions in transcription efficiency and fitness costs that are associated with these rifampicin resistance mutations (MAISNIER-PATIN AND ANDERSSON 2004). Second, we found 3 independent

examples of parallel evolution in RNA polymerase at an amino acid level (Table 1). The only gene that is not a subunit of RNA polymerase and yet showed parallel evolution in resistant populations was *cupE6*, which is involved in fimbrial biogenesis (GIRAUD *et al.* 2011). It is unclear if these mutations in *cupE6* are compensatory or generally beneficial. However, the rarity of *cupE6* mutations in our dataset for resistant populations (n = 3 mutations) suggests that they are unlikely to be a major mechanism for compensatory adaptation even if they were not generally beneficial mutations.

To test the hypothesis that low fitness costs constrain compensatory adaptation, we tested for a negative correlation between the frequencies of RNA polymerase mutations that evolved during the selection experiment and the initial cost associated with each resistant mutant (Figure 3). In support of our hypothesis, we found a significant negative correlation between the number of compensatory mutations per genome and the initial cost associated with resistance (Spearman's rank correlation: $\rho = -0.786$, $r^2 = 0.618$, $F_{1,6} = 9.72$, $P = 0.0206$). This correlation is driven by the fact that almost all clones from populations that were founded by very costly resistance mutants, such as H531R and H531Y, carry compensatory mutations, whereas no compensatory mutations were found in populations that were founded by low-cost resistance mutations such as S536F.

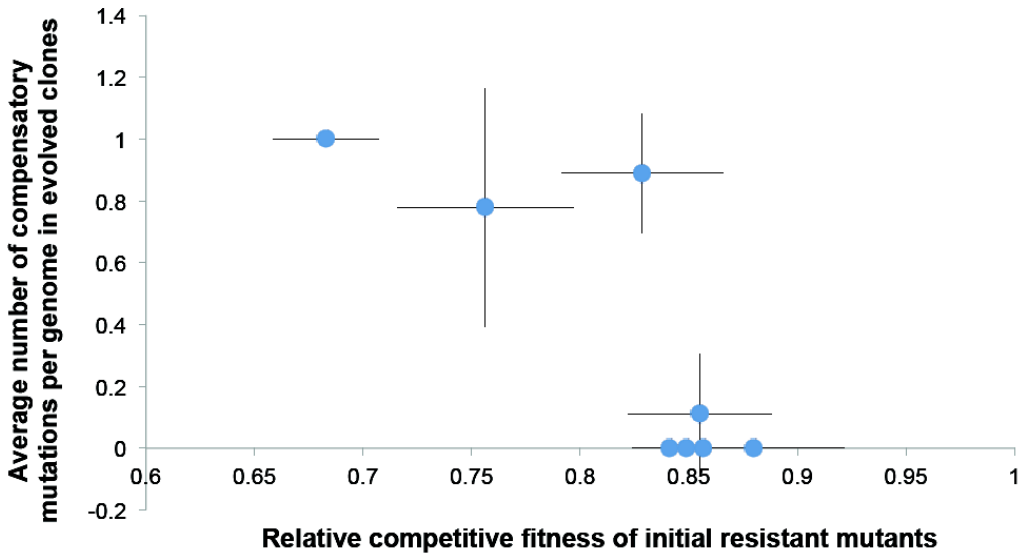


Figure 3 High fitness costs drive compensatory adaptation in rifampicin-resistant populations of *P. aeruginosa*. This graph shows the number of compensatory mutations in RNA polymerase per evolved clone (mean ± s.e; n=3) as a function of fitness cost associated with the 8 rifampicin resistance mutations (mean ± s.e; n=3). We included the reversion mutation in this analysis, because it is an adaptation to the cost of rifampicin resistance. The mean number of compensatory mutations per clone increases with the cost of resistance, as judged by a Spearman's rank-order correlation ($P < 0.05$).

Adaptation in antibiotic resistant populations is dominated by generally beneficial mutations

Although we found evidence for compensatory adaptation when the cost of resistance was large, the dominant genetic mechanism of adaptation in antibiotic resistant populations was mutations in *lasR*, an important transcriptional regulator gene involved in quorum sensing (SCHUSTER *et al.* 2003; SCHUSTER *et al.* 2004). 75% of the sequenced clones from resistant populations carried a mutation in *lasR*, including 26 independently evolved SNPs and short indel mutations, as well as 3 independent large deletions (> 3 kb) spanning *lasR*. All these missense, nonsense or frameshift mutations are expected to disrupt LasR function and result in a quorum-sensing-deficient phenotype (HEURLIER *et al.* 2005; SMITH *et al.* 2006; LUJÁN *et al.* 2007; SANDOZ *et al.* 2007; JANSEN *et al.* 2015). As an example, the missense mutation A231V in *lasR*, which was observed in two independent populations founded by the *rpoB* mutant Q518R, is known to disrupt LasR functions and key quorum-sensing regulated traits (LUJÁN *et*

al. 2007).

Such high frequency of parallel evolution in *lasR* suggests that mutations in this gene play a key role in adaptation in rifampicin-resistant populations. Consistent with this idea, we found that mutations in *lasR* lead to large increases in fitness in resistant genotypes (Figure S1, Supplementary Materials).

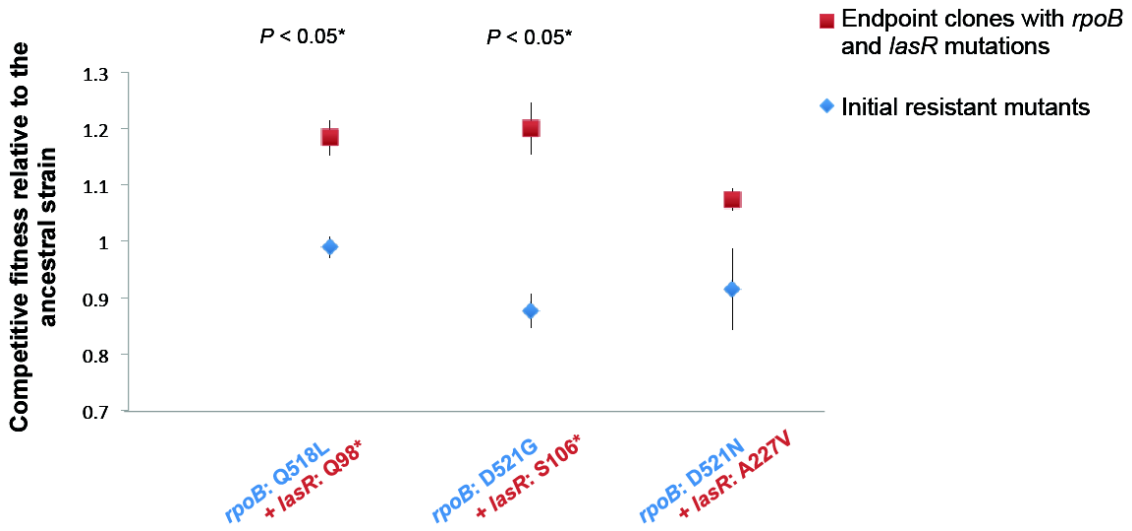


Figure S1 *lasR* mutations are beneficial. The competitive fitness (mean ± s.e; n=3) of three initial *rpoB* mutants (blue diamonds) and three evolved endpoint clones (red squares) with single point mutations in the *lasR* gene were determined relative to the rifampicin-sensitive ancestral strain. Stop-gain mutations in *lasR* are denoted by the * symbol. All three evolved endpoint clones showed an increase in average competitive fitness compared to their ancestral *rpoB* mutants, two of which are statistically significant (two-sample *t*-test, $P < 0.05$).

lasR mutations were also detected in the evolved rifampicin-sensitive control populations, implying that mutations in this gene were not compensatory. In agreement with this, numerous studies have shown that *P. aeruginosa* populations adapt to novel environments through the loss of LasR function (HEURLIER *et al.* 2005; SMITH *et al.* 2006; D'ARGENIO *et al.* 2007; SANDOZ *et al.* 2007; JANSEN *et al.* 2015). Likewise, the *lasR* mutations we found in control populations also compromise LasR function, for example by eliminating the start codon (M1I) or by deleting the *lasR* promoter region (ALBUS *et al.* 1997; KIRATISIN *et al.* 2002). However, the frequency of *lasR* mutations in control populations was lower compared

to that in the resistant populations. We found 4 independent *lasR* mutations, and only 14% of all evolved sensitive clones carried *lasR* mutations. It remains to be understood why such discrepancies in the frequencies of *lasR* mutations exist between the resistant and control populations.

The evolutionary dynamics of general and compensatory adaptation

To better understand the conflicts that existed between general adaptation and compensatory adaptation in rifampicin-resistant populations, we selected random clones from days 18 and 24 of the selection experiment in four different rifampicin resistant populations for whole-genome sequencing (Figure 4). For this analysis, we deliberately focussed on populations founded by resistant mutants carrying intermediate-cost *rpoB* mutations. Based on the sequencing results, we constructed the evolutionary history for these four populations. A key insight that emerged from our phylogenetic inference was that general adaptation, most typically through the loss of quorum sensing, tends to precede the emergence of compensatory mutations during the course of the selection experiment. In 3 of the 4 populations, general adaptation initially occurred through the spread of *lasR* mutations during the first 24 days of the experiments (Panels B-D, Figure 4). Successful *lasR* mutants then acquired compensatory mutations in RNA polymerase genes, which began to increase to detectable frequency by the end of the experiment (day 30). In the H531Y-A population, compensatory adaptation and general adaptation occurred in parallel, demonstrating clonal inference between co-existing lineages carrying beneficial and compensatory mutations in this population (Panel A, Figure 4). Initial adaptation in this population was dominated by the spread of mutations in the *gacA* gene. The GacS/GacA two-component regulatory system modulates the expression of more than 500 genes of the RsmA regulon and is known to be indirectly involved in the regulation of quorum sensing (REIMMANN *et al.* 1997; BRENCIC AND LORY 2009; RUTHERFORD AND BASSLER 2012). However, clones carrying compensatory mutations in the

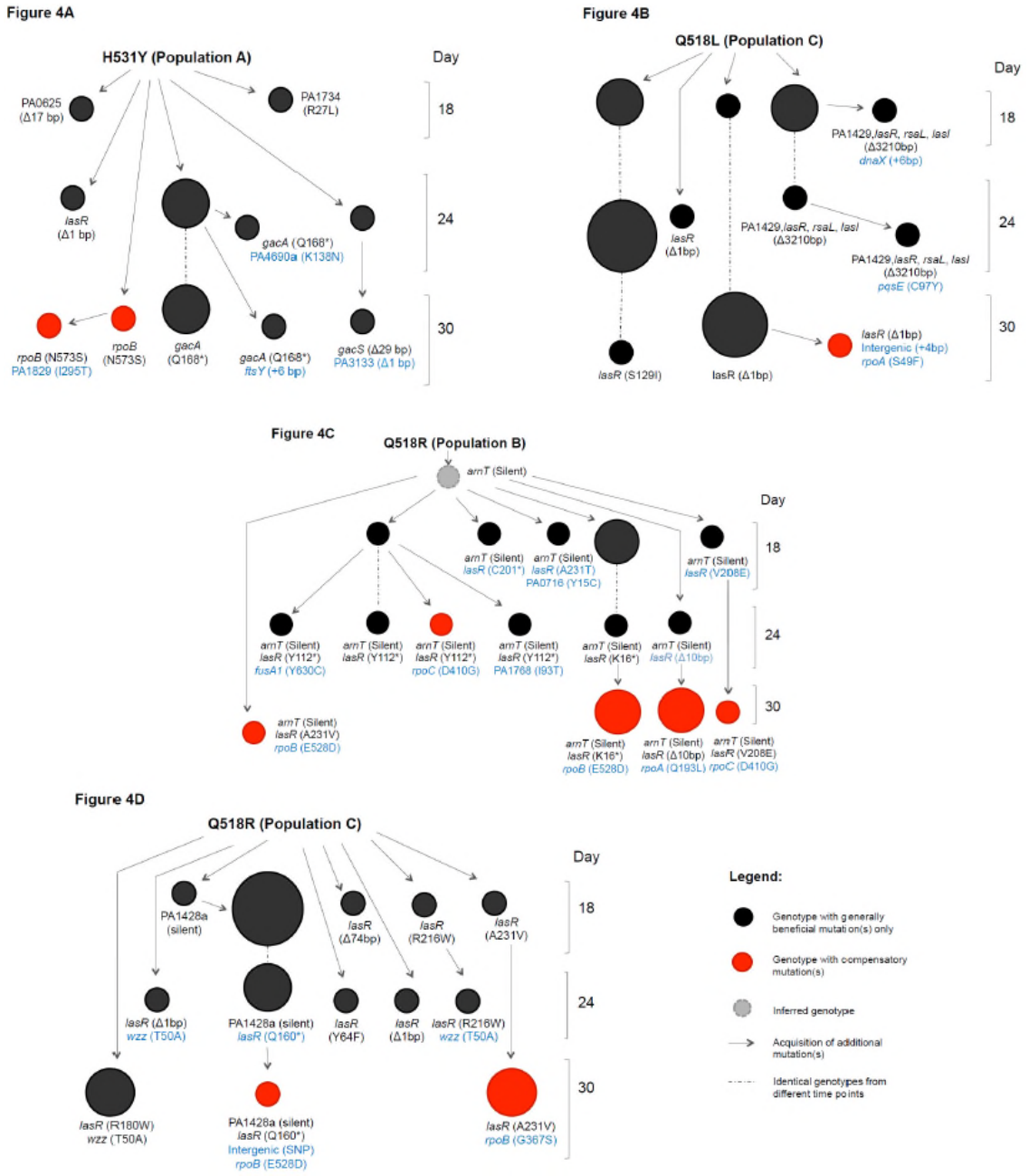


Figure 4 Clonal interference constrains compensatory adaptation. A schematic diagram representing the phylogeny inferred for six randomly selected clones sampled from the 18th, 24th and 30th (final) day from 4 populations that were polymorphic for compensatory mutations. Circles show observed genotypes, and the diameter of a circle indicates the frequency of that clone in the population (n = 1, 2 or 3). Red circles denote clones carrying compensatory mutations, while black circles denote clones carrying generally beneficial mutations, but not compensatory mutations. Each thin arrow represents the acquisition of one or more additional mutations with respect to the previous genotype. Mutations carried by individual clones are shown in text, with newly acquired mutations in blue and previously acquired mutations in black.

rpoB gene also began to increase to detectable frequency in this population by day 30.

Several factors are likely to have favoured this repeatable evolutionary dynamic of general adaptation followed by compensatory adaptation. Our whole-genome sequencing revealed that a vast diversity of beneficial mutations exists in *lasR*, and the most likely explanation is that any mutation that compromises LasR production and the quorum sensing phenotype is beneficial under the experimental conditions of this study. In contrast, we found a relatively small number of compensatory mutations in RNA polymerase, which supports the idea that compensatory mutations are rare (DAVIS *et al.* 2009). This asymmetry is clearly illustrated by the observation that the frequency parallel evolution in RNA polymerase (72.0% of clones carry identical mutations that independently evolved on >1 occasion) is much higher than that in *lasR* (6.5% of clones carrying identical *lasR* mutations that evolved >1 time) amongst the resistant populations. *lasR* mutations also confer large fitness benefits. For example, 3 double mutants that carry single *lasR* mutations in addition to their original *rpoB* mutations, showed an increased fitness of between 15.6% and 32.3% (Figure S1, Supplementary Materials). This suggests that the fitness benefit associated with *lasR* mutations is likely to have been comparable to, or greater than, the benefit associated with compensatory mutations, except when the cost of resistance was very large.

Surprisingly, we found that clones from control populations often carried mutations in genes that were not mutated in resistant populations (Figure 2), including two genes from the *wsp* operon (*wspA* and *wspF*, n = 9), *dipA* (phosphodiesterase, n = 12), and *morA* (motility regulator, n = 2). The absence of mutations in these genes in the resistant populations suggests that the fitness benefit conferred by mutations in these genes may be contingent on the presence of resistance mutations, a phenomenon known as sign epistasis (WEINREICH *et al.* 2005). In order to test this hypothesis, we measured the fitness of clones carrying *rpoB* mutations and mutations in *dipA* or *morA*. In every case, we found that *dipA* and *morA* mutations were beneficial in the presence of rifampicin resistance mutations (Heilbron *et al.*,

in preparation), implying that sign epistasis cannot explain the absence of mutations in these genes in the evolved resistant clones.

Conclusion

The idea that compensatory adaptation eliminates the cost of resistance and allows resistance alleles to effectively persist in bacterial populations has become engrained in evolutionary models of antibiotic resistance (LEVIN *et al.* 2000; ANDERSSON AND HUGHES 2010; MACLEAN *et al.* 2010a; SCHULZ ZUR WIESCH *et al.* 2010; ZUR WIESCH *et al.* 2011; MACLEAN AND VOGWILL 2014). Here we examine the underlying genomic basis of adaptation in rifampicin-resistant populations of the pathogenic bacterium *P. aeruginosa*. Although rifampicin-resistant populations quickly evolve to overcome the fitness cost associated with resistance, compensatory mutations were only fixed in populations founded by highly costly resistant mutants, as expected from principles of population genetics. Importantly, many previous studies of evolution in antibiotic resistant populations have tended to focus on evolution in populations carrying very costly resistance mutations (SCHRAG AND PERROT 1996; BJÖRKMAN *et al.* 2000; NAGAEV *et al.* 2001; MAISNIER-PATIN *et al.* 2002; KUGELBERG *et al.* 2005; NILSSON *et al.* 2006). Our results suggest that the role of compensatory mutations in overcoming the fitness cost of resistance may be less important than currently thought.

Although compensatory adaptation provides a simple solution to the cost of resistance, two constraints are likely to restrict compensatory evolution. First, generally beneficial mutations are far more common than compensatory mutations. This asymmetry is relatively intuitive to understand: compensatory mutations usually occur in the same protein or pathway as resistance mutations (MAISNIER-PATIN AND ANDERSSON 2004; DAVIS *et al.* 2009), whereas generally beneficial mutations are expected occur at many sites in the genome. In our study, this asymmetry is highlighted by the fact that repeated substitutions were common in genes involved in general adaptation, notably in *lasR*, whereas repeated mutations were much

more common in RNA polymerase genes that are involved in compensatory adaptation. Second, generally beneficial mutations can have large effects on fitness that can surpass those associated with compensatory mutations (Figure S1, Supplementary Materials). We argue that compensatory adaptation only occurred when the cost of resistance was large, because compensatory mutations provide very large fitness benefits in populations carrying costly resistance mutations.

As we have shown that the spread of compensatory mutations can be delayed by the spread of generally beneficial mutations due to clonal interference, selection for compensatory adaptation will be relatively ineffective under conditions where selection at other sites in the genome is strong. A number of important selective forces are likely to exert strong and recurrent selective pressure on populations of bacterial pathogens, such as antibiotic use (MWANGI *et al.* 2007; TOPRAK *et al.* 2012), bacteriophage (BUCKLING AND RAINEY 2002) and the immune system (SUZUKI *et al.* 2004), suggesting that compensatory adaptation may be difficult to acquire in clinical settings. These constraints are probably especially important in opportunistic pathogens, because they are essentially invading a novel niche that imposes distinct selective pressures when they establish infections in human hosts (LIEBERMAN *et al.* 2011; YOUNG *et al.* 2012; LIEBERMAN *et al.* 2014). In this respect, it is interesting to note that the best-characterized example of compensatory adaptation in a clinical setting, rifampicin resistance in *M. tuberculosis* (COMAS *et al.* 2012; BRANDIS AND HUGHES 2013; DE VOS *et al.* 2013), comes from an obligate pathogen that is well adapted to life in human hosts (SASSETTI AND RUBIN 2003).

Whole-genome sequencing is now being used extensively to study the population biology of bacterial pathogens, and it is often speculated that mutations that tend to be found in antibiotic resistant lineages are compensatory mutations (MWANGI *et al.* 2007; NIEMANN *et al.* 2009; BILLAL *et al.* 2011; CASALI *et al.* 2012; COMAS *et al.* 2012). Although there are some excellent examples of compensatory adaptation in clinical pathogens (NAGAEV *et al.* 2001;

SJÖLUND *et al.* 2003; KARAMI *et al.* 2008; LÖFMARK *et al.* 2008; COMAS *et al.* 2012; DE VOS *et al.* 2013), it is also apparent that compensatory evolution is by no means ubiquitous (MACLEAN AND VOGWILL 2014). Our study highlights some of the obstacles to evolution by compensatory mutations, but the consequences of this constraint remain unclear. On the one hand, it is possible that rapid adaptation by generally beneficial mutations effectively eliminates selection against antibiotic resistant pathogen lineages. In this scenario, resistance alleles continue to carry a cost, but selection against resistant lineages is offset by the beneficial mutations carried at other sites in the genome. Importantly, antibiotic use results in a large increase in the population size of antibiotic resistant lineages, suggesting that they should enjoy an increased likelihood of acquiring generally beneficial mutations relative to sensitive strains that are suppressed by antibiotic use. Compensatory mutations typically have epistatic interactions with resistance mutations, such that the loss of resistance mutations becomes deleterious once compensatory mutations have been acquired. By delaying the spread of compensatory mutations, it is possible that generally beneficial mutations increase the window of opportunity for reversion to antibiotic sensitivity. It is hoped that future work will shed light on the role that selection for generally beneficial mutations plays in the maintenance of resistance.

Materials and Methods

Selection experiment

The eight rifampicin-resistant *rpoB* mutants used in the selection experiment were evolved from the rifampicin-sensitive PAO1::mini-Tn7-p*LAC-lux* ancestral strain using a fluctuation test, as described in our previous work (QI *et al.* 2014). All strains were streaked on M9KB agar plates (MACLEAN *et al.* 2010b) and incubated overnight at 30°C. On the next day, 3 independent colonies per *rpoB* mutant strain were randomly selected for propagating 3 independent populations (denoted A-C) during the selection experiment. 1 ml of M9KB liquid

culture medium (MACLEAN *et al.* 2010b) was inoculated with a colony in the absence of rifampicin. The bacterial cultures were incubated overnight with shaking (225 rpm) at 30°C. On the next day, 1 µl of each overnight culture was diluted 1000-fold in fresh M9KB medium and incubated overnight under the same experimental conditions. The serial transfer was repeated for 30 consecutive days, or approximately 300 generations. Glycerol stocks of all populations were prepared on the 18th, 24th and 30th (final) day of the selection experiment. In a separate set of control experiment, 12 independent colonies of the rifampicin-sensitive PAO1::mini-Tn7-p*LAC-lux* ancestral strain were randomly selected for propagating 12 independent populations (denoted A-L) using the same experimental procedures as described above.

Competitive fitness assay

The competitive fitness of the endpoint populations of each of the propagated populations was quantified relative to the rifampicin-sensitive PAO1::mini-Tn7-pLAC-lux ancestral strain as previously described (QI *et al.* 2014). Briefly, overnight cultures of the endpoint populations, the PAO1::mini-Tn7-p*LAC-lux* ancestral strain and a GFP-tagged derivative strain of PAO1 were diluted 1:10 in M9KB medium and re-grown to early exponential phase at 30°C with continuous shaking (225 rpm). Each non-fluorescent strain was mixed with the GFP-tagged strain, diluted 200-fold in M9KB medium and incubated in Nunc 96-well microplates (Thermo Scientific, USA) at 30°C overnight for approximately 24 hours with continuous shaking. The proportion of fluorescent and non-fluorescent cells in each co-culture was determined using an Accuri C6 flow cytometer (BD Biosciences, USA) before and after the incubation. The competitive fitness of each non-fluorescent strain was calculated as the ratio of population doublings of each endpoint population relative to the GFP-tagged control strain it was competing against (HEILBRON *et al.* 2014). The relative competitive fitness of the endpoint populations was obtained by standardising their competitive fitness to that of the ancestral strain within each set of competition experiments. Three biological and three

technical replicates of each strain were assayed for each endpoint population.

To demonstrate that *lasR* mutations are beneficial under the same conditions of the selection experiment, the competitive fitness of 3 evolved rifampicin-resistant clones that carried different *lasR* mutations and the initial *rpoB* mutant strains was determined relative to the rifampicin-sensitive ancestral strain using a modified version of the competition assays, in which all strains were re-grown to exponential phase and co-cultured overnight with the GFP-tagged control strain in 5 ml Falcon tubes (BD Biosciences, USA) using exactly the same growth conditions as those under which the selection experiment was carried out.

Whole-genome sequencing

The initial *rpoB* mutants and the endpoint populations of the selection experiment were streaked on M9KB agar plates and incubated overnight at 30°C. On the next day, 3 colonies of each population were randomly selected to inoculate M9KB liquid medium incubated at 30°C with shaking overnight. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN, Netherlands) according to the manufacturer's protocol for Gram-negative bacteria. The 12 initial clones of the rifampicin-sensitive ancestral strain and the 3 randomly selected colonies from the 12 endpoint populations, which were evolved from these initial clones during the control experiment, were prepared for whole-genome sequencing using the same procedures.

To search for evidence of clonal interference in *rpoB* mutant populations, 6 random clones from the 18^{th} and 24^{th} day of the selection experiment were selected for whole-genome sequencing from four selected *rpoB* mutant populations (H531Y-A, Q518L-C, Q518R-B and Q518R-C) using the same procedures as described above. With the exception of one random clone from the endpoint population of Q518R-C (which was not successfully sequenced), 3 additional random clones from the 4 endpoint populations were whole-genome sequenced to ensure that a total of 6 clones were sequenced at each time point.

Paired-end whole-genome sequencing with read length of 100 bp was performed using the HiSeq2000 Sequencing System (Illumina, USA) at the Welcome Trust Centre for Human Genetics, University of Oxford. The average coverage was 44.1x (median coverage: 43.5x). Variants were identified using an experimentally validated in-house pipeline (HEILBRON *et al.* 2014; SAN MILLAN *et al.* 2014). The initial *rpoB* mutant isolates were found to have the same genetic background, with the exception of the specified mutations in *rpoB*. In the control experiment, the same genetic background was found in all the 12 initial ancestral strain isolates. We discarded mutations that were already present in all the initial clones with respect to the *P. aeruginosa* PAO1 reference genome, analyzing only those mutations that accumulated throughout the selection experiment.

Statistics

All statistical analyses were performed using JMP Software Version 11 (SAS). Unless otherwise stated, all statistical tests are two-tailed, and the level of significance is 0.05. The degrees of freedom are reported as subscripts next to the test statistics.

Acknowledgements

This work was supported by funding from the European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013)/ERC grant (StG-2011-281591) and from the Royal Society. We thank the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics funded by Wellcome Trust grant reference 090532/Z/09/Z and Medical Research Council Hub grant G0900747 91070 for generation of the high-throughput sequencing data. We also thank Dr. Wook Kim and members of the MacLean group for their suggestions.

Author Contributions

Conceived and designed the study: QQ, MTR, KH, GMP, RCM

Performed the experiments: QQ

Analysed the whole-genome sequencing data: MTR

Analysed the experimental data (excluding whole-genome sequencing data): QQ, KH, RCM

Wrote the paper: QQ, RCM with suggestions from MTR, KH and GMP

Competing Financial Interests

The authors declare that there are no competing financial interests.

References

Albus, A. M., E. C. Pesci, L. J. Runyen-Janecky, S. E. West and B. H. Iglewski, 1997 Vfr controls quorum sensing in Pseudomonas aeruginosa. J Bacteriol 179**:** 3928-3935.

Andersson, D. I., 2006 The biological cost of mutational antibiotic resistance: any practical conclusions? Curr Opin Microbiol 9**:** 461-465.

Andersson, D. I., and D. Hughes, 2010 Antibiotic resistance and its cost: is it possible to reverse resistance? Nat Rev Microbiol 8**:** 260-271.

Andersson, D. I., and B. R. Levin, 1999 The biological cost of antibiotic resistance. Curr Opin Microbiol 2**:** 489-493.

Bailey, S. F., A. Hinz and R. Kassen, 2014 Adaptive synonymous mutations in an experimentally evolved Pseudomonas fluorescens population. Nat Commun 5**:** 4076.

Bank, C., R. T. Hietpas, A. Wong, D. N. Bolon and J. D. Jensen, 2014 A Bayesian MCMC Approach To Assess the Complete Distribution of Fitness Effects of New Mutations: Uncovering the Potential for Adaptive Walks in Challenging Environments. Genetics.

Barrett, R. D., R. C. MacLean and G. Bell, 2006 Mutations of intermediate effect are responsible for adaptation in evolving Pseudomonas fluorescens populations. Biol Lett 2**:** 236-238.

Barrick, J. E., M. R. Kauth, C. C. Strelioff and R. E. Lenski, 2010 Escherichia coli rpoB mutants have increased evolvability in proportion to their fitness defects. Mol Biol Evol 27**:** 1338-1347.

Barrick, J. E., D. S. Yu, S. H. Yoon, H. Jeong, T. K. Oh *et al.*, 2009 Genome evolution and adaptation in a long-term experiment with Escherichia coli. Nature 461**:** 1243-1247.

Bateman, A., 1959 The viability of near-normal irradiated chromosomes. Int J Radiat Biol 1**:** 170-180.

Bell, G., 2008 *Selection: the mechanism of evolution*. Oxford University Press.

Benjamini, Y., and Y. Hochberg, 1995 Controlling the False Discovery Rate: A Practical and

Powerful Approach to Multiple Testing. J Roy Stat Soc B Met 57**:** 289-300.

Billal, D. S., J. Feng, P. Leprohon, D. Légaré and M. Ouellette, 2011 Whole genome analysis of linezolid resistance in Streptococcus pneumoniae reveals resistance and compensatory mutations. BMC Genomics 12**:** 512.

Björkman, J., I. Nagaev, O. G. Berg, D. Hughes and D. I. Andersson, 2000 Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance. Science 287**:** 1479-1482.

Boeva, V., T. Popova, K. Bleakley, P. Chiche, J. Cappo *et al.*, 2012 Control-FREEC: a tool for assessing copy number and allelic content using next-generation sequencing data. Bioinformatics 28**:** 423-425.

Brandis, G., and D. Hughes, 2013 Genetic characterization of compensatory evolution in strains carrying rpoB Ser531Leu, the rifampicin resistance mutation most frequently found in clinical isolates. J Antimicrob Chemother 68**:** 2493-2497.

Brandis, G., M. Wrande, L. Liljas and D. Hughes, 2012 Fitness-compensatory mutations in rifampicin-resistant RNA polymerase. Mol Microbiol 85**:** 142-151.

Brencic, A., and S. Lory, 2009 Determination of the regulon and identification of novel mRNA targets of Pseudomonas aeruginosa RsmA. Mol Microbiol 72**:** 612-632.

Buckling, A., and P. B. Rainey, 2002 The role of parasites in sympatric and allopatric host diversification. Nature 420**:** 496-499.

Casali, N., V. Nikolayevskyy, Y. Balabanova, O. Ignatyeva, I. Kontsevaya *et al.*, 2012 Microevolution of extensively drug-resistant tuberculosis in Russia. Genome Res 22**:** 735-745.

Centers for Disease Control and Prevention, 2013 Antibiotic resistance threats in the United States, 2013, pp., US Department of Health and Human Services.

Charlesworth, B., and K. A. Hughes, 1996 Age-specific inbreeding depression and components of genetic variance in relation to the evolution of senescence. Proc Natl Acad Sci U S A

93: 6140-6145.

Charlesworth, B., and K. A. Hughes, 2000 The maintenance of genetic variation in life-history traits, pp. 369-392 in *Evolutionary genetics: from molecules to morphology*. Cambridge University Press, Cambridge, UK.

Chen, K., J. W. Wallis, M. D. McLellan, D. E. Larson, J. M. Kalicki *et al.*, 2009 BreakDancer: an algorithm for high-resolution mapping of genomic structural variation. Nat Methods 6: 677-681.

Choi, K. H., J. B. Gaynor, K. G. White, C. Lopez, C. M. Bosio *et al.*, 2005 A Tn7-based broad-range bacterial cloning and expression system. Nat Methods 2: 443-448.

Choi, K. H., and H. P. Schweizer, 2006 mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa*. Nat Protoc 1: 153-161.

Chou, H. H., H. C. Chiu, N. F. Delaney, D. Segrè and C. J. Marx, 2011 Diminishing returns epistasis among beneficial mutations decelerates adaptation. Science 332: 1190-1192.

Choy, W. K., L. Zhou, C. K. Syn, L. H. Zhang and S. Swarup, 2004 MorA defines a new class of regulators affecting flagellar development and biofilm formation in diverse Pseudomonas species. J Bacteriol 186: 7221-7228.

Cingolani, P., A. Platts, l. L. Wang, M. Coon, T. Nguyen *et al.*, 2012 A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. Fly (Austin) 6: 80-92.

Coleman, J. R., D. Papamichail, S. Skiena, B. Futcher, E. Wimmer *et al.*, 2008 Virus attenuation by genome-scale changes in codon pair bias. Science 320: 1784-1787.

Comas, I., S. Borrell, A. Roetzer, G. Rose, B. Malla *et al.*, 2012 Whole-genome sequencing of rifampicin-resistant Mycobacterium tuberculosis strains identifies compensatory mutations in RNA polymerase genes. Nat Genet 44: 106-110.

Couce, A., J. R. Guelfo and J. Blazquez, 2013 Mutational spectrum drives the rise of mutator bacteria. PLoS Genet 9: e1003167.

Couce, A., and O. A. Tenaillon, 2015 The rule of declining adaptability in microbial evolution experiments. Front Genet 6**:** 99.

D'Argenio, D. A., M. Wu, L. R. Hoffman, H. D. Kulasekara, E. Déziel *et al.*, 2007 Growth phenotypes of Pseudomonas aeruginosa lasR mutants adapted to the airways of cystic fibrosis patients. Mol Microbiol 64**:** 512-533.

Danecek, P., A. Auton, G. Abecasis, C. A. Albers, E. Banks *et al.*, 2011 The variant call format and VCFtools. Bioinformatics 27**:** 2156-2158.

Darwin, C., 1859 *The Origin of Species*. John Murray, London.

Davies, E. K., A. D. Peters and P. D. Keightley, 1999 High frequency of cryptic deleterious mutations in *Caenorhabditis elegans*. Science 285**:** 1748-1751.

Davis, B. H., A. F. Poon and M. C. Whitlock, 2009 Compensatory mutations are repeatable and clustered within proteins. Proc Biol Sci 276**:** 1823-1827.

de Visser, J. A., T. F. Cooper and S. F. Elena, 2011 The causes of epistasis. Proc Biol Sci 278**:** 3617-3624.

de Visser, J. A., and S. F. Elena, 2007 The evolution of sex: empirical insights into the roles of epistasis and drift. Nat Rev Genet 8**:** 139-149.

de Visser, J. A., S. C. Park and J. Krug, 2009 Exploring the effect of sex on empirical fitness landscapes. Am Nat 174 Suppl 1**:** S15-30.

de Vos, M., B. Müller, S. Borrell, P. A. Black, P. D. van Helden *et al.*, 2013 Putative compensatory mutations in the rpoC gene of rifampin-resistant Mycobacterium tuberculosis are associated with ongoing transmission. Antimicrob Agents Chemother 57**:** 827-832.

Denver, D. R., D. K. Howe, L. J. Wilhelm, C. A. Palmer, J. L. Anderson *et al.*, 2010 Selective sweeps and parallel mutation in the adaptive recovery from deleterious mutation in Caenorhabditis elegans. Genome Res 20**:** 1663-1671.

DePristo, M. A., E. Banks, R. Poplin, K. V. Garimella, J. R. Maguire *et al.*, 2011 A framework for

variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet 43**:** 491-498.

Dickinson, W. J., 2008 Synergistic fitness interactions and a high frequency of beneficial changes among mutations accumulated under relaxed selection in *Saccharomyces cerevisiae*. Genetics 178**:** 1571-1578.

Drake, J. W., 1991 A constant rate of spontaneous mutation in DNA-based microbes. Proc Natl Acad Sci U S A 88**:** 7160-7164.

Durão, P., S. Trindade, A. Sousa and I. Gordo, 2015 Multiple Resistance at No Cost: Rifampicin and Streptomycin a Dangerous Liaison in the Spread of Antibiotic Resistance. Mol Biol Evol.

Elena, S. F., and R. E. Lenski, 1997 Test of synergistic interactions among deleterious mutations in bacteria. Nature 390**:** 395-398.

Estes, S., and M. Lynch, 2003 Rapid fitness recovery in mutationally degraded lines of Caenorhabditis elegans. Evolution 57**:** 1022-1030.

Estes, S., P. C. Phillips and D. R. Denver, 2011 Fitness recovery and compensatory evolution in natural mutant lines of C. elegans. Evolution 65**:** 2335-2344.

Eyre-Walker, A., and P. D. Keightley, 2007 The distribution of fitness effects of new mutations. Nat Rev Genet 8**:** 610-618.

Farhat, M. R., B. J. Shapiro, K. J. Kieser, R. Sultana, K. R. Jacobson *et al.*, 2013 Genomic analysis identifies targets of convergent positive selection in drug-resistant Mycobacterium tuberculosis. Nat Genet 45**:** 1183-1189.

Fisher, R., 1930 *The Genetical Theory of Natural Selection*. Oxford University Press, Oxford.

García-Dorado, A., 1997 The Rate and Effects Distribution of Viability Mutation in *Drosophila*: Minimum Distance Estimation. Evolution 51**:** 1130-1139.

Gerrish, P. J., and R. E. Lenski, 1998 The fate of competing beneficial mutations in an asexual population. Genetica 102-103**:** 127-144.

Gifford, D. R., and R. C. MacLean, 2013 Evolutionary reversals of antibiotic resistance in experimental populations of Pseudomonas aeruginosa. Evolution 67**:** 2973-2981.

Gifford, D. R., S. E. Schoustra and R. Kassen, 2011 The length of adaptive walks is insensitive to starting fitness in Aspergillus nidulans. Evolution 65**:** 3070-3078.

Giraud, C., C. S. Bernard, V. Calderon, L. Yang, A. Filloux *et al.*, 2011 The PprA-PprB two-component system activates CupE, the first non-archetypal Pseudomonas aeruginosa chaperone-usher pathway system assembling fimbriae. Environ Microbiol 13**:** 666-683.

Govan, J. R., A. R. Brown and A. M. Jones, 2007 Evolving epidemiology of Pseudomonas aeruginosa and the Burkholderia cepacia complex in cystic fibrosis lung infection. Future Microbiol 2**:** 153-164.

Haldane, J., 1937 The effect of variation of fitness. American Naturalist**:** 337-349.

Hall, A. R., V. F. Griffiths, R. C. MacLean and N. Colegrave, 2010 Mutational neighbourhood and mutation supply rate constrain adaptation in Pseudomonas aeruginosa. Proc Biol Sci 277**:** 643-650.

Hall, A. R., J. C. Iles and R. C. MacLean, 2011 The fitness cost of rifampicin resistance in Pseudomonas aeruginosa depends on demand for RNA polymerase. Genetics 187**:** 817-822.

Halligan, D. L., and P. D. Keightley, 2009 Spontaneous mutation accumulation studies in evolutionary genetics. Annu Rev Ecol Evol S 40**:** 151-172.

Hammerschmidt, K., C. J. Rose, B. Kerr and P. B. Rainey, 2014 Life cycles, fitness decoupling and the evolution of multicellularity. Nature 515**:** 75-79.

Heilbron, K., D. Gifford and R. MacLean, in preparation The genomics of adaptation to mutational degradation in hypermutator *Pseudomonas aeruginosa*.

Heilbron, K., M. Toll-Riera, M. Kojadinovic and R. C. MacLean, 2014 Fitness is strongly influenced by rare mutations of large effect in a microbial mutation accumulation experiment. Genetics 197**:** 981-990.

Heurlier, K., V. Dénervaud, M. Haenni, L. Guy, V. Krishnapillai *et al.*, 2005 Quorum-sensing-negative (lasR) mutants of Pseudomonas aeruginosa avoid cell lysis and death. J Bacteriol 187**:** 4875-4883.

Hughes, D., and D. I. Andersson, 2015 Evolutionary consequences of drug resistance: shared principles across diverse targets and organisms. Nat Rev Genet 16**:** 459-471.

Hughes, K. A., 2010 Mutation and the evolution of ageing: from biometrics to system genetics. Philos Trans R Soc Lond B Biol Sci 365**:** 1273-1279.

Huse, H. K., T. Kwon, J. E. Zlosnik, D. P. Speert, E. M. Marcotte *et al.*, 2010 Parallel evolution in *Pseudomonas aeruginosa* over 39,000 generations in vivo. MBio 1.

Imhof, M., and C. Schlotterer, 2001a Fitness effects of advantageous mutations in evolving *Escherichia coli*. Proc Natl Acad Sci U S A 98**:** 1113-1117.

Imhof, M., and C. Schlotterer, 2001b Fitness effects of advantageous mutations in evolving Escherichia coli populations. Proc Natl Acad Sci U S A 98**:** 1113-1117.

Jansen, G., L. L. Crummenerl, F. Gilbert, T. Mohr, R. Pfefferkorn *et al.*, 2015 Evolutionary Transition from Pathogenicity to Commensalism: Global Regulator Mutations Mediate Fitness Gains through Virulence Attenuation. Mol Biol Evol.

Jerome, J. P., J. A. Bell, A. E. Plovanich-Jones, J. E. Barrick, C. T. Brown *et al.*, 2011 Standing Genetic Variation in Contingency Loci Drives the Rapid Adaptation of *Campylobacter jejuni* to a Novel Host. PLoS ONE 6**:** e16399.

Joseph, S. B., and D. W. Hall, 2004 Spontaneous mutations in diploid *Saccharomyces cerevisiae*: more beneficial than expected. Genetics 168**:** 1817-1825.

Karami, N., C. Hannoun, I. Adlerberth and A. E. Wold, 2008 Colonization dynamics of ampicillin-resistant Escherichia coli in the infantile colonic microbiota. J Antimicrob Chemother 62**:** 703-708.

Keightley, P. D., 1994 The distribution of mutation effects on viability in *Drosophila melanogaster*. Genetics 138**:** 1315-1322.

Khan, A. I., D. M. Dinh, D. Schneider, R. E. Lenski and T. F. Cooper, 2011 Negative epistasis between beneficial mutations in an evolving bacterial population. Science 332**:** 1193-1196.

Kimura, M., 1983 The neutral theory of molecular evolution, pp., Cambridge.

Kimura, M., 1985 The role of compensatory neutral mutations in molecular evolution. Journal of Genetics 64**:** 7-19.

Kiratisin, P., K. D. Tucker and L. Passador, 2002 LasR, a transcriptional activator of Pseudomonas aeruginosa virulence genes, functions as a multimer. J Bacteriol 184**:** 4912-4919.

Klockgether, J., A. Munder, J. Neugebauer, C. F. Davenport, F. Stanke *et al.*, 2010 Genome diversity of Pseudomonas aeruginosa PAO1 laboratory strains. J Bacteriol 192**:** 1113-1121.

Kondrashov, A. S., 1988 Deleterious mutations and the evolution of sexual reproduction. Nature 336**:** 435-440.

Kryazhimskiy, S., D. P. Rice and M. M. Desai, 2012 Population subdivision and adaptation in asexual populations of Saccharomyces cerevisiae. Evolution 66**:** 1931-1941.

Kryazhimskiy, S., D. P. Rice, E. R. Jerison and M. M. Desai, 2014 Microbial evolution. Global epistasis makes adaptation predictable despite sequence-level stochasticity. Science 344**:** 1519-1522.

Kugelberg, E., S. Löfmark, B. Wretlind and D. I. Andersson, 2005 Reduction of the fitness burden of quinolone resistance in Pseudomonas aeruginosa. J Antimicrob Chemother 55**:** 22-30.

Lee, H., E. Popodi, H. Tang and P. L. Foster, 2012 Rate and molecular spectrum of spontaneous mutations in the bacterium *Escherichia coli* as determined by whole-genome sequencing. Proc Natl Acad Sci U S A 109**:** E2774-2783.

Lenski, R. E., and M. Travisano, 1994 Dynamics of adaptation and diversification: a 10,000-

generation experiment with bacterial populations. Proc Natl Acad Sci U S A 91**:** 6808-6814.

Levin, B. R., V. Perrot and N. Walker, 2000 Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. Genetics 154**:** 985-997.

Li, H., and R. Durbin, 2009 Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25**:** 1754-1760.

Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan *et al.*, 2009 The Sequence Alignment/Map format and SAMtools. Bioinformatics 25**:** 2078-2079.

Lieberman, T. D., K. B. Flett, I. Yelin, T. R. Martin, A. J. McAdam *et al.*, 2014 Genetic variation of a bacterial pathogen within individuals with cystic fibrosis provides a record of selective pressures. Nat Genet 46**:** 82-87.

Lieberman, T. D., J. B. Michel, M. Aingaran, G. Potter-Bynoe, D. Roux *et al.*, 2011 Parallel bacterial evolution within multiple patients identifies candidate pathogenicity genes. Nat Genet 43**:** 1275-1280.

Lin, W. H., and E. Kussell, 2012 Evolutionary pressures on simple sequence repeats in prokaryotic coding regions. Nucleic Acids Res 40**:** 2399-2413.

Llamas, M. A., M. J. Mooij, M. Sparrius, C. M. Vandenbroucke-Grauls, C. Ratledge *et al.*, 2008 Characterization of five novel Pseudomonas aeruginosa cell-surface signalling systems. Mol Microbiol 67**:** 458-472.

Löfmark, S., C. Jernberg, H. Billström, D. I. Andersson and C. Edlund, 2008 Restored fitness leads to long-term persistence of resistant Bacteroides strains in the human intestine. Anaerobe 14**:** 157-160.

Long, H. A., T. Paixão, R. B. Azevedo and R. A. Zufall, 2013 Accumulation of Spontaneous Mutations in the Ciliate Tetrahymena thermophila. Genetics.

Luján, A. M., A. J. Moyano, I. Segura, C. E. Argaraña and A. M. Smania, 2007 Quorum-sensing-deficient (lasR) mutants emerge at high frequency from a Pseudomonas aeruginosa

mutS strain. Microbiology 153: 225-237.

Lynch, M., 2010 Evolution of the mutation rate. Trends Genet 26: 345-352.

Lynch, M., W. Sung, K. Morris, N. Coffey, C. R. Landry *et al.*, 2008 A genome-wide view of the spectrum of spontaneous mutations in yeast. Proc Natl Acad Sci U S A 105: 9272-9277.

MacLean, R. C., and A. Buckling, 2009 The distribution of fitness effects of beneficial mutations in Pseudomonas aeruginosa. PLoS Genet 5: e1000406.

MacLean, R. C., A. R. Hall, G. G. Perron and A. Buckling, 2010a The population genetics of antibiotic resistance: integrating molecular mechanisms and treatment contexts. Nat Rev Genet 11: 405-414.

MacLean, R. C., G. G. Perron and A. Gardner, 2010b Diminishing returns from beneficial mutations and pervasive epistasis shape the fitness landscape for rifampicin resistance in Pseudomonas aeruginosa. Genetics 186: 1345-1354.

MacLean, R. C., and T. Vogwill, 2014 Limits to compensatory adaptation and the persistence of antibiotic resistance in pathogenic bacteria. Evol Med Public Health 2015: 4-12.

Maisnier-Patin, S., and D. I. Andersson, 2004 Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. Res Microbiol 155: 360-369.

Maisnier-Patin, S., O. G. Berg, L. Liljas and D. I. Andersson, 2002 Compensatory adaptation to the deleterious effect of antibiotic resistance in Salmonella typhimurium. Mol Microbiol 46: 355-366.

Maisnier-Patin, S., J. R. Roth, A. Fredriksson, T. Nyström, O. G. Berg *et al.*, 2005 Genomic buffering mitigates the effects of deleterious mutations in bacteria. Nat Genet 37: 1376-1379.

Mandsberg, L. F., M. D. Macia, K. R. Bergmann, L. E. Christiansen, M. Alhede *et al.*, 2011 Development of antibiotic resistance and up-regulation of the antimutator gene. FEMS Microbiol Lett 324: 28-37.

Marco-Sola, S., M. Sammeth, R. Guigo and P. Ribeca, 2012 The GEM mapper: fast, accurate and versatile alignment by filtration. Nat Methods 9**:** 1185-1188.

Martin, G., and T. Lenormand, 2006 A general multivariate extension of Fisher's geometrical model and the distribution of mutation fitness effects across species. Evolution 60**:** 893-907.

Marvig, R. L., H. K. Johansen, S. Molin and L. Jelsbak, 2013 Genome Analysis of a Transmissible Lineage of *Pseudomonas aeruginosa* Reveals Pathoadaptive Mutations and Distinct Evolutionary Paths of Hypermutators. PLoS Genet 9**:** e1003741.

Mavrodi, D. V., A. Joe, O. V. Mavrodi, K. A. Hassan, D. M. Weller *et al.*, 2011 Structural and functional analysis of the type III secretion system from Pseudomonas fluorescens Q8r1-96. J Bacteriol 193**:** 177-189.

Melnyk, A. H., A. Wong and R. Kassen, 2015 The fitness costs of antibiotic resistance mutations. Evol Appl 8**:** 273-283.

Michener, J. K., A. A. Camargo Neves, S. Vuilleumier, F. Bringel and C. J. Marx, 2014 Effective use of a horizontally-transferred pathway for dichloromethane catabolism requires post-transfer refinement. Elife 3.

Miller, J. H., 1996 Spontaneous mutators in bacteria: insights into pathways of mutagenesis and repair. Annu Rev Microbiol 50**:** 625-643.

Mira, A., and N. A. Moran, 2002 Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria. Microb Ecol 44**:** 137-143.

Miralles, R., P. J. Gerrish, A. Moya and S. F. Elena, 1999 Clonal interference and the evolution of RNA viruses. Science 285**:** 1745-1747.

Moore, F., and R. Woods, 2006 Tempo and constraint of adaptive evolution in *Escherichia coli*. Biological Journal of the Linnean Society 88**:** 403–411.

Moran, N. A., H. J. McLaughlin and R. Sorek, 2009 The dynamics and time scale of ongoing genomic erosion in symbiotic bacteria. Science 323**:** 379-382.

Moxon, R., C. Bayliss and D. Hood, 2006 Bacterial contingency loci: the role of simple sequence DNA repeats in bacterial. Annu Rev Genet 40**:** 307-333.

Mukai, T., 1964 The genetic structure of natural populations of *Drosophila melanogaster*. I. Spontaneous mutation rate of polygenes controlling viability. Genetics 50**:** 1-19.

Mwangi, M. M., S. W. Wu, Y. Zhou, K. Sieradzki, H. de Lencastre *et al.*, 2007 Tracking the in vivo evolution of multidrug resistance in Staphylococcus aureus by whole-genome sequencing. Proc Natl Acad Sci U S A 104**:** 9451-9456.

Nagaev, I., J. Björkman, D. I. Andersson and D. Hughes, 2001 Biological cost and compensatory evolution in fusidic acid-resistant Staphylococcus aureus. Mol Microbiol 40**:** 433-439.

Nahum, J. R., P. Godfrey-Smith, B. N. Harding, J. H. Marcus, J. Carlson-Stevermer *et al.*, 2015 A tortoise-hare pattern seen in adapting structured and unstructured populations suggests a rugged fitness landscape in bacteria. Proc Natl Acad Sci U S A 112**:** 7530-7535.

Ness, R. W., A. D. Morgan, N. Colegrave and P. D. Keightley, 2012 Estimate of the spontaneous mutation rate in *Chlamydomonas reinhardtii*. Genetics 192**:** 1447-1454.

Niemann, S., C. U. Köser, S. Gagneux, C. Plinke, S. Homolka *et al.*, 2009 Genomic diversity among drug sensitive and multidrug resistant isolates of Mycobacterium tuberculosis with identical DNA fingerprints. PLoS One 4**:** e7407.

Nilsson, A. I., A. Zorzet, A. Kanth, S. Dahlström, O. G. Berg *et al.*, 2006 Reducing the fitness cost of antibiotic resistance by amplification of initiator tRNA genes. Proc Natl Acad Sci U S A 103**:** 6976-6981.

Oliver, A., 2010 Mutators in cystic fibrosis chronic lung infection: Prevalence, mechanisms, and consequences for antimicrobial therapy. Int J Med Microbiol 300**:** 563-572.

Oliver, A., R. Cantón, P. Campo, F. Baquero and J. Blázquez, 2000 High frequency of hypermutable Pseudomonas aeruginosa in cystic fibrosis lung infection. Science 288**:** 1251-1254.

Orr, H. A., 2002 The population genetics of adaptation: the adaptation of DNA sequences. Evolution 56**:** 1317-1330.

Orsi, R. H., B. M. Bowen and M. Wiedmann, 2010 Homopolymeric tracts represent a general regulatory mechanism in prokaryotes. BMC Genomics 11**:** 102.

Otto, S. P., and M. C. Whitlock, 1997 The probability of fixation in populations of changing size. Genetics 146**:** 723-733.

Partridge, L., and N. H. Barton, 1993 Optimality, mutation and the evolution of ageing. Nature 362**:** 305-311.

Patel, R. K., and M. Jain, 2012 NGS QC Toolkit: a toolkit for quality control of next generation sequencing data. PLoS One 7**:** e30619.

Perdigão, J., H. Silva, D. Machado, R. Macedo, F. Maltez *et al.*, 2014 Unraveling Mycobacterium tuberculosis genomic diversity and evolution in Lisbon, Portugal, a highly drug resistant setting. BMC Genomics 15**:** 991.

Perfeito, L., L. Fernandes, C. Mota and I. Gordo, 2007 Adaptive mutations in bacteria: high rate and small effects. Science 317**:** 813-815.

Perfeito, L., A. Sousa, T. Bataillon and I. Gordo, 2014 Rates of fitness decline and rebound suggest pervasive epistasis. Evolution 68**:** 150-162.

Phillips, P. C., 2008 Epistasis--the essential role of gene interactions in the structure and evolution of genetic systems. Nat Rev Genet 9**:** 855-867.

Poon, A., B. H. Davis and L. Chao, 2005 The coupon collector and the suppressor mutation: estimating the number of compensatory mutations by maximum likelihood. Genetics 170**:** 1323-1332.

Qi, Q., G. M. Preston and R. C. MacLean, 2014 Linking system-wide impacts of RNA polymerase mutations to the fitness cost of rifampin resistance in Pseudomonas aeruginosa. MBio 5**:** e01562.

Qi, Q., M. Toll-Riera, K. Heilbron and R. MacLean, in preparation The genomic basis of

adaptation to the fitness cost of rifampicin resistance in *Pseudomonas aeruginosa*.

R Core Team, 2015 R: A language and environment for statistical computing, pp. R Foundation for Statistical Computing, Vienna, Austria.

R Development Core Team, 2012 R: A language and environment for statistical computing, pp. R Foundation for Statistical Computing, Vienna, Austria.

Rahme, L. G., E. J. Stevens, S. F. Wolfort, J. Shao, R. G. Tompkins *et al.*, 1995 Common virulence factors for bacterial pathogenicity in plants and animals. Science 268**:** 1899-1902.

Reimmann, C., M. Beyeler, A. Latifi, H. Winteler, M. Foglino *et al.*, 1997 The global activator GacA of Pseudomonas aeruginosa PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. Mol Microbiol 24**:** 309-319.

Reynolds, M. G., 2000 Compensatory evolution in rifampin-resistant Escherichia coli. Genetics 156**:** 1471-1481.

Rice, P., I. Longden and A. Bleasby, 2000 EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet 16**:** 276-277.

Rozen, D. E., J. A. de Visser and P. J. Gerrish, 2002 Fitness effects of fixed beneficial mutations in microbial populations. Curr Biol 12**:** 1040-1045.

Rozen, D. E., N. Philippe, J. Arjan de Visser, R. E. Lenski and D. Schneider, 2009 Death and cannibalism in a seasonal environment facilitate bacterial coexistence. Ecol Lett 12**:** 34-44.

Rutherford, S. T., and B. L. Bassler, 2012 Bacterial quorum sensing: its role in virulence and possibilities for its control. Cold Spring Harb Perspect Med 2.

San Millan, A., R. Peña-Miller, M. Toll-Riera, Z. V. Halbert, A. R. McLean *et al.*, 2014 Positive selection and compensatory adaptation interact to stabilize non-transmissible plasmids. Nat Commun 5**:** 5208.

Sandoz, K. M., S. M. Mitzimberg and M. Schuster, 2007 Social cheating in Pseudomonas

aeruginosa quorum sensing. Proc Natl Acad Sci U S A 104: 15876-15881.

Sanjuán, R., A. Moya and S. F. Elena, 2004 The contribution of epistasis to the architecture of fitness in an RNA virus. Proc Natl Acad Sci U S A 101: 15376-15379.

Sassetti, C. M., and E. J. Rubin, 2003 Genetic requirements for mycobacterial survival during infection. Proc Natl Acad Sci U S A 100: 12989-12994.

Schrag, S. J., and V. Perrot, 1996 Reducing antibiotic resistance. Nature 381: 120-121.

Schrag, S. J., V. Perrot and B. R. Levin, 1997 Adaptation to the fitness costs of antibiotic resistance in Escherichia coli. Proc Biol Sci 264: 1287-1291.

Schulz zur Wiesch, P., J. Engelstädter and S. Bonhoeffer, 2010 Compensation of fitness costs and reversibility of antibiotic resistance mutations. Antimicrob Agents Chemother 54: 2085-2095.

Schuster, M., C. P. Lostroh, T. Ogi and E. P. Greenberg, 2003 Identification, timing, and signal specificity of Pseudomonas aeruginosa quorum-controlled genes: a transcriptome analysis. J Bacteriol 185: 2066-2079.

Schuster, M., M. L. Urbanowski and E. P. Greenberg, 2004 Promoter specificity in Pseudomonas aeruginosa quorum sensing revealed by DNA binding of purified LasR. Proc Natl Acad Sci U S A 101: 15833-15839.

Segrè, A. V., A. W. Murray and J. Y. Leu, 2006 High-resolution mutation mapping reveals parallel experimental evolution in yeast. PLoS Biol 4: e256.

Shaw, F. H., C. J. Geyer and R. G. Shaw, 2002 A comprehensive model of mutations affecting fitness and inferences for *Arabidopsis thaliana*. Evolution 56: 453-463.

Shaw, R. G., D. L. Byers and E. Darmo, 2000 Spontaneous mutational effects on reproductive traits of *Arabidopsis thaliana*. Genetics 155: 369-378.

Silander, O. K., O. Tenaillon and L. Chao, 2007 Understanding the evolutionary fate of finite populations: the dynamics of mutational effects. PLoS Biol 5: e94.

Sjölund, M., K. Wreiber, D. I. Andersson, M. J. Blaser and L. Engstrand, 2003 Long-term

persistence of resistant Enterococcus species after antibiotics to eradicate Helicobacter pylori. Ann Intern Med 139**:** 483-487.

Smit, A., R. Hubley and P. Green, 1996-2010 *RepeatMasker Open-3.0*, pp.

Smith, E. E., D. G. Buckley, Z. Wu, C. Saenphimmachak, L. R. Hoffman *et al.*, 2006 Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A 103**:** 8487-8492.

Stern, D. L., 2013 The genetic causes of convergent evolution. Nat Rev Genet 14**:** 751-764.

Stevens, K. E., and M. E. Sebert, 2011 Frequent beneficial mutations during single-colony serial transfer of *Streptococcus pneumoniae*. PLoS Genet 7**:** e1002232.

Sung, W., M. S. Ackerman, S. F. Miller, T. G. Doak and M. Lynch, 2012a Drift-barrier hypothesis and mutation-rate evolution. Proc Natl Acad Sci U S A 109**:** 18488-18492.

Sung, W., A. E. Tucker, T. G. Doak, E. Choi, W. K. Thomas *et al.*, 2012b Extraordinary genome stability in the ciliate *Paramecium tetraurelia*. Proc Natl Acad Sci U S A 109**:** 19339-19344.

Suzuki, K., B. Meek, Y. Doi, M. Muramatsu, T. Chiba *et al.*, 2004 Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proc Natl Acad Sci U S A 101**:** 1981-1986.

Szamecz, B., G. Boross, D. Kalapis, K. Kovács, G. Fekete *et al.*, 2014 The genomic landscape of compensatory evolution. PLoS Biol 12**:** e1001935.

Szendro, I., M. Schenk, J. Franke, J. Krug and J. de Visser, 2013 Quantitative analyses of empirical fitness landscapes. Journal of Statistical Mechanics: Theory and Experiment P01005.

Tatusov, R. L., M. Y. Galperin, D. A. Natale and E. V. Koonin, 2000 The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Res 28**:** 33-36.

Tenaillon, O., A. Rodríguez-Verdugo, R. L. Gaut, P. McDonald, A. F. Bennett *et al.*, 2012 The molecular diversity of adaptive convergence. Science 335**:** 457-461.

Toprak, E., A. Veres, J. B. Michel, R. Chait, D. L. Hartl *et al.*, 2012 Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. Nat Genet 44**:** 101-105.

Torres-Barcelo, C., G. Cabot, A. Oliver, A. Buckling and R. C. Maclean, 2013 A trade-off between oxidative stress resistance and DNA repair plays a role in the evolution of elevated mutation rates in bacteria. Proc Biol Sci 280**:** 20130007.

Treves, D. S., S. Manning and J. Adams, 1998 Repeated evolution of an acetate-crossfeeding polymorphism in long-term populations of Escherichia coli. Mol Biol Evol 15**:** 789-797.

Trindade, S., L. Perfeito and I. Gordo, 2010 Rate and effects of spontaneous mutations that affect fitness in mutator *Escherichia coli*. Philos Trans R Soc Lond B Biol Sci 365**:** 1177-1186.

Vogwill, T., M. Kojadinovic and R. MacLean, in preparation Epistatic interactions between antibiotic resistance mutations and genetic background shape the fitness cost of antibiotic resistance across species of *Pseudomonas*.

Vogwill, T., and R. C. MacLean, 2015 The genetic basis of the fitness costs of antimicrobial resistance: a meta-analysis approach. Evol Appl 8**:** 284-295.

Weinreich, D. M., N. F. Delaney, M. A. Depristo and D. L. Hartl, 2006 Darwinian evolution can follow only very few mutational paths to fitter proteins. Science 312**:** 111-114.

Weinreich, D. M., Y. Lan, C. S. Wylie and R. B. Heckendorn, 2013 Should evolutionary geneticists worry about higher-order epistasis? Curr Opin Genet Dev 23**:** 700-707.

Weinreich, D. M., R. A. Watson and L. Chao, 2005 Perspective: Sign epistasis and genetic constraint on evolutionary trajectories. Evolution 59**:** 1165-1174.

Wichman, H. A., L. A. Scott, C. D. Yarber and J. J. Bull, 2000 Experimental evolution recapitulates natural evolution. Philos Trans R Soc Lond B Biol Sci 355**:** 1677-1684.

Wielgoss, S., J. E. Barrick, O. Tenaillon, S. Cruveiller, B. Chane-Woon-Ming *et al.*, 2011 Mutation Rate Inferred From Synonymous Substitutions in a Long-Term Evolution Experiment

With Escherichia coli. G3 (Bethesda) 1**:** 183-186.

Winsor, G. L., D. K. Lam, L. Fleming, R. Lo, M. D. Whiteside *et al.*, 2011 Pseudomonas Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. Nucleic Acids Res 39**:** D596-600.

Woods, R. J., J. E. Barrick, T. F. Cooper, U. Shrestha, M. R. Kauth *et al.*, 2011 Second-order selection for evolvability in a large Escherichia coli population. Science 331**:** 1433-1436.

Wright, S., 1932 The roles of mutation, inbreeding, crossbreeding, and selection in evolution. Proceedings of the Sixth International Congress of Genetics 1**:** 356–366.

Ye, K., M. H. Schulz, Q. Long, R. Apweiler and Z. Ning, 2009 Pindel: a pattern growth approach to detect break points of large deletions and medium sized insertions from paired-end short reads. Bioinformatics 25**:** 2865-2871.

Young, B. C., T. Golubchik, E. M. Batty, R. Fung, H. Larner-Svensson *et al.*, 2012 Evolutionary dynamics of Staphylococcus aureus during progression from carriage to disease. Proc Natl Acad Sci U S A 109**:** 4550-4555.

Zar, J. H., 2010 *Biostatistical analysis*. Pearson Prentice-Hall, Upper Saddle River, NJ, USA.

Zhang, L., and L. T. Watson, 2009 Analysis of the fitness effect of compensatory mutations. HFSP J 3**:** 47-54.

Zorzet, A., M. Y. Pavlov, A. I. Nilsson, M. Ehrenberg and D. I. Andersson, 2010 Error-prone initiation factor 2 mutations reduce the fitness cost of antibiotic resistance. Mol Microbiol 75**:** 1299-1313.

zur Wiesch, P. A., R. Kouyos, J. Engelstädter, R. R. Regoes and S. Bonhoeffer, 2011 Population biological principles of drug-resistance evolution in infectious diseases. Lancet Infect Dis 11**:** 236-247.

Zwickl, D., 2006 Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion, pp., Ph.D. dissertation, The University of Texas at Austin.