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Mutational neighbourhood and mutation supply rate constrain adaptation in *Pseudomonas aeruginosa*

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Understanding adaptation by natural selection requires understanding the genetic factors that determine which beneficial mutations are available for selection. Here, using experimental evolution of rifampicin-resistant *Pseudomonas aeruginosa*, we show that different genotypes vary in their capacity for adaptation to the cost of antibiotic resistance. We then use sequence data to show that the beneficial mutations associated with fitness recovery were specific to particular genetic backgrounds, suggesting that genotypes had access to different sets of beneficial mutations. When we manipulated the supply rate of beneficial mutations, by altering effective population size during evolution, we found that it constrained adaptation in some selection lines by restricting access to rare beneficial mutations, but that the effect varied among the genotypes in our experiment. These results suggest that mutational neighbourhood varies even among genotypes that differ by a single amino acid change, and this determines their capacity for adaptation as well as the influence of population biology processes that alter mutation supply rate.

**Keywords**: compensatory adaptation; mutational neighbourhood; *Pseudomonas aeruginosa*; experimental evolution

1. INTRODUCTION

During adaptation to a novel selection pressure, the evolutionary trajectory of a population will depend on both its mutational neighbourhood (Burch & Chao 2000) and the rate at which beneficial mutations are supplied relative to population biology processes that lead to the stochastic loss of rare alleles. First, the set of beneficial mutations that are accessible (mutational neighbourhood) and the distribution of their fitness effects will determine the potential for adaptation. Second, the product of the frequency of beneficial mutations and effective population size (mutation supply rate) will determine the way in which the mutational neighbourhood is sampled. For example, in a very large population with a high rate of beneficial mutation, all possible mutations may be present in every generation and the next mutation to fix is likely to be one that confers a relatively large increase in fitness. Conversely, when the population is small or has a low average rate of beneficial mutation, the next mutation to fix will be the first one that, by chance, is not stochastically lost when it is rare.

In this paper, we examine the importance of both mutational neighbourhood and mutation supply rate during adaptation of bacteria to the fitness costs associated with antibiotic resistance. Mutations that confer antibiotic resistance often incur a cost when selecting drugs are withdrawn, and bacteria can evolve to ameliorate these costs by compensatory mutations at other sites that have epistatic effects specific to particular resistance mutations (Cohan et al. 1994; Schrag et al. 1997; Maisnier-Patin & Andersson 2004). Compensatory adaptation may help to explain the persistence of resistant bacteria in clinical settings (Andersson & Levin 1999; Bergstrom & Feldgarden 2008). Therefore, understanding the genetic factors that determine the spread of beneficial mutations in pathogenic populations is directly relevant to the global problem of resistance. To manipulate mutational neighbourhood, we used a range of genotypes with different resistance mutations on the same gene. Access to beneficial mutations has been shown to vary among genotypes, and to constrain adaptation in both RNA viruses (Burch & Chao 2000) and in bacteria carrying deleterious mutations (Moore et al. 2000). For example, recent work has shown that different synonymous variants of the same gene adapt to a novel selection pressure by different amino acid substitutions (Cambray & Mazel 2008). We therefore expected that the rate of adaptation, and the mutational pathway by which it proceeded, would vary among genotypes carrying different resistance mutations.

To study the importance of mutation supply rate, we manipulated the effective population size of evolving populations by changing the dilution factor during serial transfer. This imposes periodic bottlenecks in population size that are expected to alter the genetic variation available for selection (Bergstrom et al. 1999; Levin et al. 2000; Elena et al. 2001; Wahl et al. 2002; Li & Roossinck 2004). Specifically, the supply of beneficial mutations...
should be relatively low under severe bottlenecks, because rare alleles are more likely to be lost before they can spread. We therefore predicted that the reduced mutation supply rate under severe bottlenecking would lead to a relatively small response to selection, and that this effect would be most pronounced in genetic backgrounds where beneficial mutations that fully recover the cost of resistance are relatively rare.

We maintained selection lines initiated with different rifampicin-resistant (Rif\(^R\)) *Pseudomonas aeruginosa* genotypes for approximately 200 generations in the absence of antibiotics, evolving each genotype at a range of bottleneck sizes. Rifampicin works by binding to a conserved domain of the \(\beta\)-subunit of RNA polymerase (RNAP), thereby blocking elongation of the RNA transcript (Severinov et al. 1993; Campbell et al. 2001). Resistance can result from any of several possible mutations on the \(rpoB\) gene that change the structure of RNAP and prevent binding (Telenti et al. 1993; Pozzi et al. 1999; Campbell et al. 2001; Trinh et al. 2006). Rif\(^R\) mutations often incur a fitness cost in terms of reduced growth rate and the magnitude of the cost varies among different mutations for *P. aeruginosa* (MacLean & Buckling 2009). At the end of the experiment we tested for variation in fitness among populations that were initiated with different Rif\(^R\) genotypes, and for the effects of bottlenecks on the response to selection. In order to identify mutations associated with increased fitness, we sequenced the central rifampicin resistance region of \(rpoB\) for populations that showed clear evidence of adaptation. We expected that any differences in mutational neighbourhood among resistant genotypes would be reflected in both the fitness of evolved populations and the identity of any associated mutations in \(rpoB\). We also predicted that the response to selection would be relatively small under tight bottlenecking.

2. MATERIAL AND METHODS

(a) Bacterial strains

We used four defined Rif\(^R\) genotypes that were isolated at random from a fluctuation test with the rifampicin-sensitive wild-type (*P. aeruginosa* PA01) on agar plates containing 62.11 \(\mu\)g ml\(^{-1}\) rifampicin (MacLean & Buckling 2009). Each Rif\(^R\) mutant carries a single mutation on \(rpoB\) and each mutation is associated with a different amino acid change in RNAP that confers resistance (MacLean & Buckling 2009). Two mutations were at a residue that comes into direct contact with rifampicin (H531R and H531Y), one was at a residue that interacts indirectly with rifampicin (S517L) and one was an insertion mutation (+P518) that may affect residues involved in both direct and indirect Rif-RNAP interactions. We refer to Rif\(^R\) genotypes below by their amino acid changes relative to the wild-type.

(b) Selection experiment

Sixty-four selection lines were grown at 37°C in 6 ml of King’s Medium B (KB) in 28 ml glass universalss with continuous shaking at 180g. Upon transfer an aliquot was transferred from each stationary phase culture to fresh media. We manipulated bottleneck size by changing the dilution factor (\(d\)), which meant transferring a different volume in each treatment. We used the following dilution factors: 0.000001, 0.0001, 0.01 and 0.1, so that the number of cells transferred ranged from approximately 15 000 at the smallest value of \(d\) to approximately 1500 million at the largest. Four replicate selection lines of each of the four Rif\(^R\) genotypes were maintained at each of the four bottleneck sizes for approximately 200 generations. We also wanted to see if changes in fitness during the experiment represented compensatory adaptation specific to the defined resistance genotypes, or general adaptation of *P. aeruginosa* to our experimental conditions. To this end we ran four selection lines with the wild-type at \(d = 0.01\). All selection lines were frozen in 50 per cent v/v glycerol at \(-80°C\) every approximately 50 generations.

Changing the dilution factor (\(d\)) also changes the number of doublings between transfers (\(n\)). This potentially confounds our manipulation of bottleneck size with the number of generations each line was selected for. To account for this we ran each selection line for 200/\(n\) transfers, calculating \(n\) as follows. Assuming growth by binary fission, the number of cells increases by a factor of 2\(^n\) for every \(n\) doublings. During serial transfer, the proportional increase in the number of cells (2\(^n\)) between transfers equals \(1/d\) (e.g. \(d = 0.01\) amounts to a 100-fold increase per transfer). If 2\(^n\) = 1/\(d\), then \(n = \ln(1/d)/\ln(2)\).

(c) Fitness assays

Fitness was calculated as growth rate relative to the wild-type (Björkman et al. 2000). We estimated growth rate as the slope of log(OD\(_{600}\)) over time during exponential growth in KB. For each assay the population was reconditioned from frozen by overnight growth in KB, then diluted in M9 salt solution and 20 \(\mu\)l inoculated into 180 \(\mu\)l of KB in a randomly assigned well of a 96-well plate. We then followed changes in OD\(_{600}\) using a SpectraMax M2 microplate reader (Molecular Devices Corporation). Following Novak et al. (2006), we corrected measurements by subtracting the scores for blank wells that were measured individually for each well, and discarding scores below the detection limit of the spectrophotometer (OD = 0.002). Growth rates were estimated from Baranyi models (Baranyi & Roberts 1994) fitted to each growth curve using the nlstools package in R 2.2.1 (R Development Core Team 2005). Four cultures of each unevolved Rif\(^R\) mutant and wild-type PA01 were tested alongside evolved populations that were frozen after 50, 100, 150 and 200 generations. Each population was tested in triplicate in each of two blocks.

(d) Resistance assays

We also tested whether evolved genotypes remained resistant to rifampicin or reverted to susceptibility. To do this we used selective plating to estimate the proportion of Rif\(^R\) and rifampicin-sensitive (Rif\(^S\)) genotypes in each population at the end of the experiment. Populations were reconditioned by overnight growth in KB, then diluted in M9 salt solution and plated onto permissive KB agar. At least 40 colonies per population (mean ± s.d. = 103.6 ± 59.7) were then transferred to KB agar with 62.11 \(\mu\)g ml\(^{-1}\) rifampicin and the proportion of resistant colonies was recorded. To compare the fitness effects of mutations that caused reversion to the wild-type and other beneficial mutations that occurred during the selection experiment, we isolated five Rif\(^R\) and five Rif\(^S\) genotypes at random from each of three polymorphic H531R populations and assayed their growth rates as above.
(e) Statistical analysis
We analysed the fitness of evolved bacteria at the end of the experiment using a linear mixed effects model (lme function in R v. 2.2.1) with RifR genotype and bottleneck size as fixed effects and block as a random effect, using the varIdent function (nlme library) to model heterogeneous variances among groups. We treated RifR genotype as a fixed factor because we chose these genotypes for their varying levels of fitness relative to the ancestor. If all four RifR genotypes fully recovered the cost of resistance, then they would reach fitness of approximately 1.0 and there would be no significant effect of genotype in the model. Similarly, if bottlenecking had the same effect for all four genotypes, then there would be no significant bottleneck size × genotype interaction.

(f) Sequencing
We sequenced approximately 500 bp of rpoB for all 16 evolved populations for each of the two RifR genotypes that showed clear evidence of compensatory adaptation (H531R and H531Y), as well as four cultures for each of their unevolved RifR ancestral genotypes and wild-type PA01. First, genomic DNA was extracted using a Wizard Genomic DNA kit (Promega). We then amplified the central rifampicin resistance region of rpoB between nucleotides 1798 and 1262 with primers rpoB_fwd (5'-GTTCTTGACCGCGCAGGCG-3') and rpoB_rev (5'-GCGATGACGGTGCCTGCGC-3'). Reaction mixtures consisted of BIOTAQ polymerase (Bioline, UK), 1 mM dNTPs, 16 nM (NH4)2SO4, 62.5 mM Tris–HCl (pH 8.8), 0.01 per cent Tween 20, 2 mM MgCl2, and each primer at a concentration of 10 pM. Amplification reactions were as follows: 94°C for 5 min, followed by 35 cycles of 94°C (30 s), 55°C (30 s), 60°C (30 s) and 72°C (60 s), before final incubation at 72°C for 10 min. PCR products were quantified on 1 per cent agarose gels and purified using ExoSAP (USB Europe). Purified PCR products were then sequenced with both forward and reverse primers using BigDye 3.1 sequencing (Applied Biosystems International), followed by ethanol/EDTA precipitation. Sequences were then edited and aligned against the International, followed by ethanol/EDTA precipitation. Control samples showed that the obtained PA01 sequence was as expected, and the H531R and H531Y sequences from the start of the experiment contained only their respective resistance mutations as identified previously (MacLean & Buckling 2009).

Four sequences showed polymorphisms at two nucleotide sites. In order to identify which of the four possible sequences were present in these samples we cloned PCR products from three of them into TOPO Vectors (Invitrogen) and transformed them into One Shot TOP10F chemically competent Escherichia coli cells. Six positive (white) colonies were then isolated for each population and cultured overnight. Plasmid DNA was then isolated with a Wizard Plus Miniprep DNA Purification kit (Promega) and sequencing reactions were carried out as above.

3. RESULTS
(a) Fitness recovery
Different genotypes varied in their capacity to recover the costs associated with rifampicin resistance (figure 1). This was reflected by variation in fitness at the end of our experiment among selection lines that were initiated with different RifR genotypes (F1,47 = 17.00, p < 0.0001). Specifically, all of the H531R and H531Y selection lines increased in fitness, reaching growth rates equal or close to that of the wild-type. By contrast, lines with S517L mutations showed little evidence of adaptation in any treatment. Most of the +P518 lines also showed no improvement, although 3 out of 16 reached fitness equal to or greater than 1.0 (figure 1). We also evolved PA01 control lines at d = 0.01, but found no significant change in growth rate at the end of the experiment (F1,2 = 2.19, p = 0.18), suggesting that adaptation in the other selection lines was owing to compensatory mutations rather than general adaptation to the experimental conditions.

(b) Do different genotypes acquire the same beneficial mutations?
Variation of the response to selection could result from different populations fixing different beneficial mutations that vary in their selective value, or from the same beneficial mutation fixing in all populations but having a different selective value in different genetic backgrounds. To discriminate between these alternatives we aimed to identify the beneficial mutations responsible for fitness recovery in H531R and H531Y populations. We detected a total of four nonsynonymous mutations in the sequenced region of rpoB: two in H531R and two in H531Y populations (see the electronic supplementary material, table S1). Two of these mutations resulted in unique amino acid sequences, with each mutation being specific to a particular genetic background (F519L was only found in populations that were initially H531R, while N573S was only found in populations that were initially H531Y). The remaining two nucleotide substitutions were also specific to one or other of the genetic backgrounds, even though they resulted in the same amino acid sequence relative to the wild-type (H531C), and did not alter rifampicin resistance. The H531R and H531Y genotypes differ from the wild-type by having different substitutions at the same codon, and can both reach a third amino acid sequence (H531C) by further mutations. However, all four substitutions were also specific to one or other of the genetic backgrounds, even though they resulted in the same amino acid sequence relative to the wild-type (H531C), and did not alter rifampicin resistance.

(c) Effect of bottlenecks on mutation supply and adaptation
Consistent with our prediction that severe bottlenecks would constrain adaptation by restricting access to rare beneficial mutations, some lines showed a reduced selection response under severe bottlenecking. However, this effect varied among the different RifR genotypes in our
For H531R lines, where we found the clearest evidence of adaptation, the response to selection was lower under severe bottlenecking ($F_{3,12} = 26.46, p < 0.0001$; figure 1). By contrast, for H531Y selection lines that also recovered the cost of resistance there was no significant effect of bottlenecking ($F_{3,12} = 0.50, p = 0.69$; figure 1).

In order to confirm that the effect of bottlenecking was owing to its influence on the beneficial mutations associated with increasing fitness, we compared the rpoB mutations in H531R and H531Y populations evolved at different bottleneck sizes. For H531R, we identified different rpoB mutations in populations evolved at different bottleneck sizes. Specifically, the H531C mutation was found in the 12 populations that evolved at the three largest bottleneck sizes, reaching fixation in 10 of those 12 (see the electronic supplementary material, table S1). At the smallest bottleneck size we identified a different mutation (F519L) at fixation in one of the four
populations. For H531Y, we detected N573S and H531C mutations at the three largest bottleneck sizes only (see the electronic supplementary material, table S1). N573S was at fixation in 2 out of 7 of the populations in which we detected it, and H531C was at fixation in 0 out of 5 populations. We were unable to identify mutations in rpoB for all four H531Y and three H531R populations at the lowest bottleneck size. Presumably adaptation in these populations was owing either to extragenic mutations or substitutions elsewhere on rpoB. Thus, selection lines adapted by different mutational pathways under severe bottlenecks, and this was associated with a relatively small response to selection for H531R but not H531Y.

**4. Discussion**

We initiated selection lines with different RifR genotypes and found that they varied in their capacity for adaptation to the cost of resistance—some genotypes rapidly recovered fitness, even at small effective population size, while others failed to recover fitness over the course of this experiment. For selection lines that showed clear evidence of fitness recovery we identified associated mutations on rpoB that were specific to particular genetic backgrounds. Furthermore, the effect of periodic bottlenecks in population size on adaptation varied between selection lines with different RifR mutations, suggesting a difference in the number and effect size of beneficial mutations that were available to different genotypes. These results provide good evidence that there is substantial variation in mutational neighbourhood among genotypes carrying different resistance mutations, and this variation had a profound effect on the potential for adaptation, the mutational pathway to higher fitness, and the influence of mutation supply rate on adaptation.

Several lines of evidence suggest that the beneficial mutations that we identified have epistatic effects, as is the case for compensatory mutations (Schrag & Perrot 1996; Burch & Chao 1999). First, the mutations that we identified are in amino acid positions in rpoB that are highly conserved among bacteria, suggesting that their beneficial effects are contingent on the presence of rifampicin resistance mutations. In support of this, we failed to find evidence of adaptation in this experiment in the rifampicin-sensitive PA01 control lines. Second, variation among RifR genotypes for both the response to selection and the mutational pathway to higher fitness suggests that the beneficial effects of compensatory mutations are specific to particular resistance genotypes. This concurs with evidence that different E. coli genotypes vary in their potential for compensatory adaptation, even when they have similar levels of fitness (Moore et al. 2000). Furthermore, Reynolds (2000) showed that different RifR E. coli genotypes can acquire different secondary mutations in rpoB. Consistent with this, we identified two amino acid changes in rpoB (F519L and N573S) that were specific to different genotypes (H531R and H531Y, respectively). Taken together, these results support the notion that genetically distinct populations of bacteria often take different mutational pathways to higher fitness during adaptation to the same novel selection pressure (Travisano et al. 1995; Riley et al. 2001; MacLean & Bell 2003; Woods et al. 2006). However, we also found that these two genotypes could converge on the same amino acid change relative to the wild-type (H531C) by acquiring a second substitution at the same residue as their resistance mutations. Thus, the specificity of the F519L and N573S mutations suggests that H531R and H531Y have different mutational neighbourhoods, even though they overlap by both having access to H531C by a single substitution.

Variation in mutational neighbourhood may also explain the different effects of bottlenecks in H531R and H531Y selection lines. Bottlenecking had the effect we predicted in H531R lines, presumably because it takes rare big-benefit compensatory mutations to recover the fitness cost completely, and under tight bottlenecking the population only has access to the most common mutations rather than the best ones. The same effect
has been observed previously in RNA viruses (Burch & Chao 1999; Sanjuán et al. 2005). By contrast, there was no effect of bottlenecks on the change in fitness for H531Y lines, suggesting that big-benefit mutations were frequent enough to be accessible even under tight bottlenecking. This could reflect a difference in the distribution of fitness effects of beneficial mutations between the two genotypes, with results for H531R being consistent with a distribution with many mutations of small effect and few mutations of large effect, such as an exponential distribution, and results from H531Y being consistent with a distribution with many mutations of equivalent effect. While the scale of our experiment limits our ability to identify the distribution of effect sizes for either genotype, variation of the effects of bottlenecking is at least consistent with their being different. A comparatively high frequency of big-benefit mutations in the mutational neighbourhood of H531Y is also consistent with our finding another beneficial mutation (N573S) in some of the same populations as H531C at larger bottleneck sizes. Interestingly, the simultaneous presence of different beneficial mutations can result in clonal interference, increasing the time taken for a given beneficial mutation to fix (Gerrish & Lenski 1998; de Visser & Rozen 2006). As we describe below, this may be linked to the relatively gradual increase in fitness for some H531Y lines compared with H531R, as well as the failure of H531C and N573S to reach fixation in several selection lines.

If H531C genotypes were not prevented from spreading by the presence of other beneficial mutations, or lost during bottlenecking, we would expect them to have fixed in all of the H531R selection lines over the 200 generations of our experiment. To demonstrate this we calculated the expected number of generations required for a single H531C genotype to approach fixation (frequency of 0.99), assuming its appearance at the beginning of the experiment. Following Nilsson et al. (2004; see also Bell 2008), the time required for an established mutant in an asexual population to reach a defined frequency is equal to $t = (1/s \ln (2)) \times \ln(F_f/F_0)$. Here $s$ is the selection coefficient, so that the mutant has fitness of $1 + s$ times that of the resident genotype; $F_f$ and $F_0$ are the final and initial ratios of the mutant to the wild-type, which we take as 99/1 and 1/bottleneck size, respectively. We estimate that a single H531C mutant present at the start of our experiment would approach fixation in a H531R population within 22–40 generations (depending on bottleneck size) unless it was lost to drift or faced competition from other beneficial mutations. This is consistent with the rapid increase in fitness for H531R selection lines, with all 16 showing the largest selection response over the first 50 generations, and the finding that H531C was at fixation in several populations at the end of the experiment. For H531Y populations, the same genotype would take longer to fix because of a smaller selection coefficient, and we would expect H531C to approach fixation within 137–248 generations if its spread was not impeded by other beneficial mutations. Thus, mutations that fully recovered the cost of resistance would not necessarily have fixed in all populations during our experiment, even if we assume that they were present instantaneously and ignore the effects of drift. Therefore, clonal interference is not required to explain the slow spread of H531C mutations in H531Y selection lines under large bottleneck sizes. However, the presence of a second beneficial mutation (N573S) in several of these populations shows that clonal interference must have occurred in at least some of these selection lines.

Our finding that different resistant genotypes take different evolutionary trajectories in the absence of antibiotics supports the view that fitness landscapes can be rugged for microbial populations that have been displaced from a fitness peak by a deleterious mutation (Burch & Chao 1999; Colegrave & Buckling 2005). Instead of recovering fitness by ascending the original peak, and thereby reverting to antibiotic susceptibility, they can reach high fitness at another peak by mutations that do not restore the original phenotype. This outcome has been repeatedly observed, with several studies showing that compensatory adaptation is more probable than reversion to susceptibility (Cohan et al. 1994; Schrag et al. 1997; Björkman et al. 1998; Moore et al. 2000; Reynolds 2000; Nagae et al. 2001; Maisnier-Patin & Andersson 2004; Kugelberg et al. 2005; Sanjuán et al. 2005; Nilsson et al. 2006; Schoustra et al. 2006; Paulander et al. 2007). This is explained partly by the relatively high frequency of compensatory mutations relative to reversion mutations (Poon et al. 2005). This mechanism is probably the main reason for the lack of reversion in our experiment. However, we also found that compensatory mutations could confer an equal fitness benefit to reversion mutations. Therefore even when the effective population size is very large, reversion mutations are unlikely to fix because of the presence of many alternative beneficial mutations with similar fitness effects. Furthermore, the tendency of both H531R and H531Y genotypes to mutate to H531C rather than the wild-type suggests that the relative rates of these particular base changes may be biased against reversion to antibiotic susceptibility. These and other results suggest that prudent use of antibiotics is important to avoid creating widespread populations of resistant bacteria that are unlikely to revert to susceptibility (Billington et al. 1999; Levin 2001; De Gelder et al. 2004) without infection controls and the immigration of susceptible bacteria.

Variation in mutational neighbourhood is attributable to two factors. First, the number of amino acid changes that can result from a single substitution at a given codon is limited, and this constrains the area of protein space that can be explored from any one genotype (Maynard Smith 1970). This principle is illustrated in our results by the H531C amino acid change, which is only attainable by double mutation from the wild-type, beginning with an H531R or H531Y mutation that is only beneficial in the presence of rifampicin. Second, a given mutation can be beneficial in one genetic background and deleterious in another (Weinreich et al. 2005), leading to variation of the mutational pathway to higher fitness among genotypes. Such epistasis is reflected in our results by the different beneficial mutations that we detected in H531R and H531Y. Moreover, the lack of compensatory adaptation for +P518 and S517L suggests that mutations that were beneficial for H531R and H531Y did not have the same effect here. This variation in the range and effect size of compensatory mutations is not surprising given that deleterious mutations can vary greatly in their effects on protein structure and function.
For example, a deleterious mutation that alters the function of a protein will have few potential compensatory mutations compared with a mutation that alters protein stability, because there are relatively few mutations that affect protein function, whereas most nonsynonymous mutations will affect protein stability (Pakula & Sauer 1989; DePristo et al. 2005). It follows that variation of mutational neighbourhoods is probably of very broad relevance, given that it derives from fundamental constraints imposed by the properties of the genetic code and the biophysical effects of deleterious mutations.

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