# Mutators drive evolution of multi-resistance to antibiotics

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## Abstract

Combination drug treatments are an approach used to counter the evolution of resistance–the guiding principle being that they can prevent multiple independent resistance mutations from arising sequentially in the same genome. Here, we show that bacterial populations with 'mutators', organisms defective in DNA repair, can evolve multi-resistance under conditions where purely wild-type populations cannot. We exposed experimental populations of *Escherichia coli* to rising concentrations of single-drug and combination antibiotic treatments. Introducing mutators at low-to-intermediate frequencies permitted the evolution of multi-resistance. Notably, the evolution of multi-resistance did not require direct selection for both resistance mutations, as the elevated mutation rates allowed it to evolve under single-drug and combination treatments alike. Using eco-evolutionary simulations, we show that hitch-hiking with single resistance mutations allowed the mutator allele to sweep to fixation. The resulting increase in mutation supply was the key to evolving multi-resistance sequentially. While simulations also demonstrated that multi-resistance can arise in large populations, the size required exceeded those typical of infection. Ultimately, our results suggest that the utility of combination therapy may be limited when mutators are present, and when achieving or maintaining therapeutic antibiotic concentrations is difficult.

## Introduction

There has been sustained interest in the use of 'combination therapy' for preventing resistance evolution in infectious disease[1–3] and cancer[4]. Where standard 'monotherapy' uses only one drug, combination therapy uses multiple drugs at the same time, an approach that has proved successful in various settings, particularly in treating HIV[5–8]. Combination therapy is currently the standard treatment against some microbial infections e.g. tuberculosis[9], but there is growing interest in deploying combination therapy more broadly to combat the global burden of antimicrobial resistance[10–12]. However, evidence for combination therapy preventing resistance evolution in microbes is limited, with some combinations faring no better than monotherapy in some contexts[13,14]. Although there is a suggestion of an advantage of combination therapy over other approaches that use multiple drugs (namely 'mixing' and 'cycling'[15,16]), this is not universal[17,18]. Discovering what governs the ability of combinations to prevent resistance

evolution is therefore a critical area of research.

Combination therapy is based on the idea that resistance to multiple antibiotics requires multiple independent resistance mutations. In general, acquiring resistance to combination therapy ('multi-resistance') should be harder than acquiring resistance to monotherapy ('single resistance'). This relies on two main assumptions. First, mutation rates to resistance are generally assumed to be small for bacteria, on the order of $10^{-7}$ to $10^{-10}$ per generation[19]. The probability of acquiring multiple independent resistance mutations during the same replication event is the product of their individual mutation rates[1,20]. For two mutations, this is on the order of $10^{-14}$ to $10^{-20}$ per generation (discounting specific combinations where resistance can be achieved by a 'multi-drug resistance' or 'cross-resistance' mechanism[21]). The emergence of multi-resistance during a single replication event is therefore assumed to be extremely rare. Second, strains with resistance mutations should only increase in frequency in environments where the mutations provide a selective advantage. Multi-resistance should not occur in environments unless multiple antibiotics are present. If multiple antibiotics are used simultaneously, they should inhibit the growth of both sensitive and single-resistant bacteria, thus multi-resistance should not emerge through mutations occurring sequentially in successive generations.

In this study, we tested these assumptions in the evolution of multi-resistance. Using a combination of experimental evolution and stochastic modelling, we challenged populations of *Escherichia coli* with evolving antibiotic resistance to rifampicin and nalidixic acid, either used singly or in combination. Populations consisted of a mixture of wild-type individuals, and 'mutator' organisms. Typical mutation rates of mutators range between 10- and 1000-fold higher than wild-type[22,23]. We varied mutator frequency from 0–30% as mutators are found at these frequencies in natural populations of bacteria, including infections and host-associated microbiomes[24–27]. Mutators are a potential concern for combination therapy, as multi-resistance is often found in mutator lineages, although this is believed to have arisen due to sequential application of monotherapy[22,28–31]. Direct evidence for the efficacy of combinations against mutators is mixed[32–34]. We imposed selection for resistance by increasing the concentrations of antibiotics over a physiologically-relevant concentration gradient[35,36]. Concentrations can vary over time and space, both in clinical settings (e.g. within-patient pharmacokinetics[37]) and nature (e.g. wastewater contamination[38] and agriculture[39]). Concentration gradients are expected to facilitate the evolution of resistance[40,41],

particularly if multiple mutations are required[42–45].

Our results demonstrate that populations with mutators at a low-to-intermediate initial frequency can evolve multi-resistance where purely wild-type populations cannot. Strikingly, mutators facilitated multi-resistance evolution not just under combination selection, where it was favoured, but also under single-drug selection. Using stochastic simulations, we determined that genetic hitch-hiking of the mutator allele alongside single-drug resistance allowed an increase mutator frequency, which enabled a subsequent resistance mutation to arise in the same genetic background as the first. Multi-resistance then swept to fixation under combination selection, but remained at a detectable frequency under single-drug selection. A difference in mutation rate between mutator and wild-type individuals, which allowed mutators to acquire sequential resistance mutations, was sufficient to explain multi-resistance evolution in these populations. Multi-resistance evolved without requiring multi-drug resistance mechanisms, cross-resistance, or spontaneous double mutations. Given the prevalence of mutators in natural populations, multi-resistance evolution may represent a considerable challenge to the wider deployment of combination therapy against bacterial infections. Moreover, because multi-resistance emerged in both single-drug and combination treatments environments, genetic variation underlying resistance to combination treatments may already be present, even before the use of such treatments becomes common pratice.

## Results

### Mutators facilitate multi-resistance evolution by sequential acquisition of independent mutations

We exposed populations of bacteria with different initial mutator frequencies to four selection regimes: two single-drug environments involving rifampicin or nalidixic acid, a combination environment with both antibiotics, and an antibiotic-free control. Antibiotic concentrations were doubled daily over six days, starting at 0.625 mg/l and ending at 20 mg/l of each drug (where 10 mg/l of either drug is sufficient to inhibit the starting strain). Figure 1 shows the number of populations with resistance in each treatment detected by selective plating. In the absence of selection for resistance, multi-resistance was not detected for any mutator frequency, although single resistance was observed. In the

absence of mutators, multi-resistance was not detected for any treatment with or without antibiotics, although single resistance was again observed transiently. However, when mutators were present, multi-resistance was observed in both of the single-drug treatments and the combination treatment. In general, nalidixic acid treatment resulted in the fewest multi-resistant populations, whereas rifampicin and combination treatment produced roughly similar numbers. The combination treatment resulted in the highest proportion of populations where no resistance was detected. In this sense, the combination treatment was more effective at suppressing resistance, even in the presence of mutators. However, a considerable proportion of populations (23.3%–61.7%) nevertheless exhibited multi-resistance when mutators were present. Single-drug treatments had predictable effects on single-drug resistance, i.e. rifampicin resistance was more likely to occur under rifampicin treatment, but not nalidixic acid treatment, and vice versa. All antibiotic treatments increased the probability of multi-resistance evolving.

The effect of mutator initial frequency and antibiotic treatment on resistance state of populations at the end of the experiment was determined using a Bayesian categorical mixed-effects model (Supplementary Information Section 2.1). A full model incorporating both mutator initial frequency and antibiotic treatment (main effects) and their interactions provided only a marginally better fit than a main effects-only model, hence we present contrasts from the latter (Supplementary Table 6). All levels of 'mutators' increased the proportion of populations exhibiting single-drug resistance (including mixed resistance) or multi-resistance. There was a positive association between initial mutator frequency and resistance; however, the treatment contrasts for each level of mutator frequency had overlapping 95% C.I.s.

## Multi-resistance evolves in single-drug treatments despite conferring no growth advantage over single-resistance

The emergence of multi-resistant strains in single-drug treatments was an unexpected finding. In searching for an explanation, we first considered whether multi-resistance conferred an advantage in single-drug environments. The growth of sensitive, single- and multi-resistant clones was assayed under all conditions experienced during experimental evolution. Growth assays were conducted on clones derived from fluctuation tests using the non-mutator wild-type BW25113, to minimise confounding effects of other mutations arising during experimental evolution (Supplementary Figure 1). For the combination

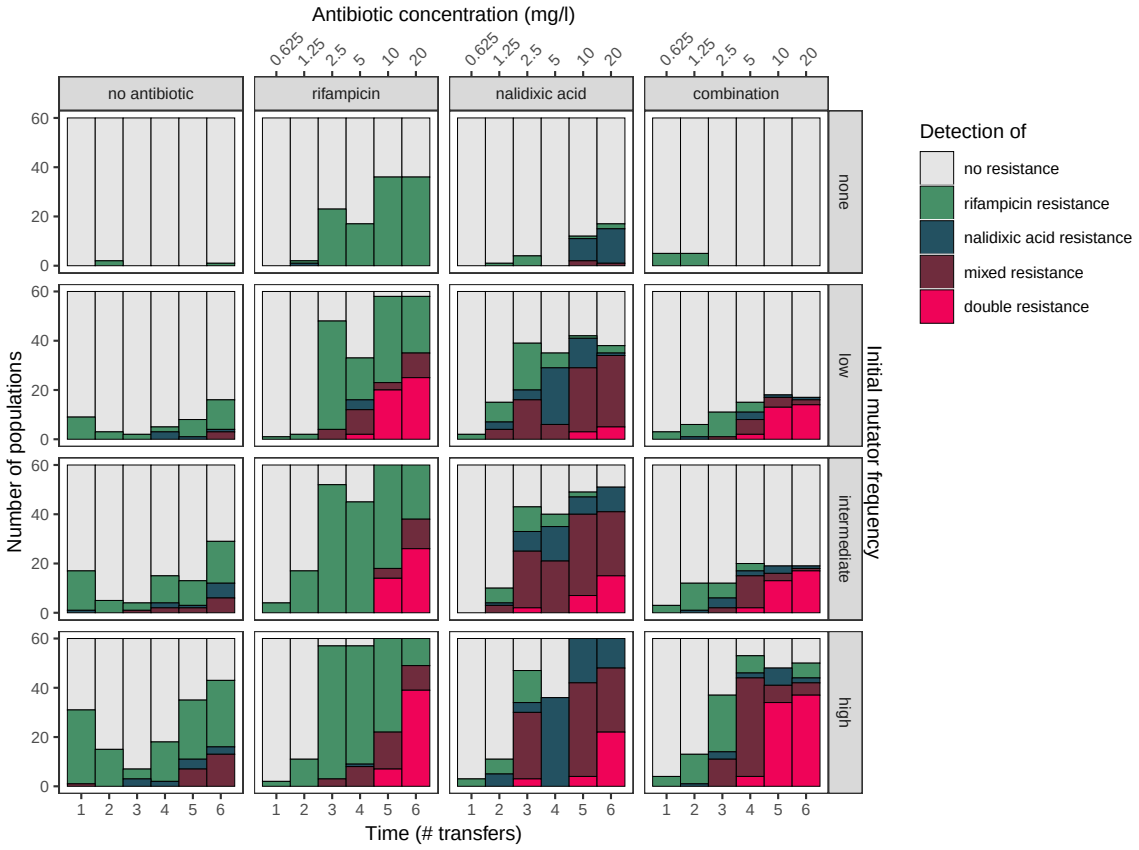


**Figure 1: Multi-resistance evolves in single-drug and combination drug treatments in populations with mutators.** Stacked bars show numbers of populations where each resistance state was detected by selective plating of a portion of the population on antibiotic-containing medium (see Methods). 'Rifampicin resistance' or 'nalidixic acid resistance' indicates growth only on the indicated antibiotic. 'Mixed resistance' indicates growth on each antibiotic separately, but not in combination (i.e. both single-resistant types present in the population). 'Multi-resistance' indicates growth on both antibiotics alone and in combination. For the associated statistical analysis, see Supplementary Information Section 2.1.

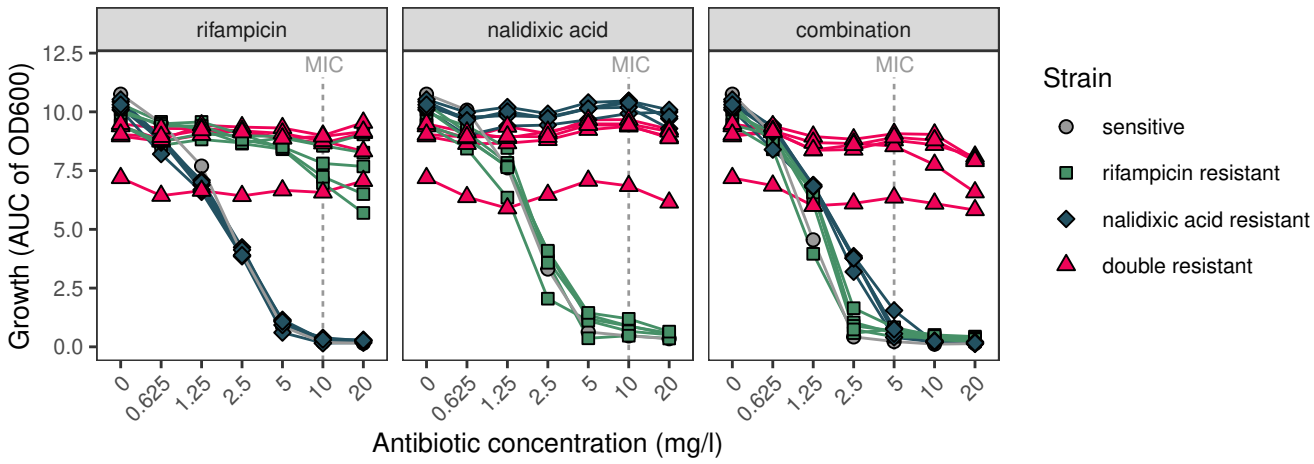


**Figure 2: Growth of sensitive, single-, and double-drug resistant strains at antibiotic concentrations experienced during the resistance selection regime** (AUC of OD600: area under curve of growth curves measuring optical density at 600 nm over time). Resistant strains ($n = 5$) were isolated in the wild-type *E. coli* K-12 BW25113 by fluctuation test(s). MIC–minimum inhibitory concentration of sensitive strain. Error bars (±SE) are smaller than symbols. AUC was calculated from the growth curves shown in Supplementary Figure 1. For the associated statistical analysis, see Supplementary Information Section 2.2.

treatment, there was a clear advantage of multi-resistance over single-drug resistance for concentrations of 1.25 mg/l and above (Figure 2). These concentrations correspond to time periods where multi-resistance begins to be detected in the experiments. In contrast, there was no benefit of multi-resistance over rifampicin resistance in the rifampicin treatment [difference in growth = -0.07, 95% C.I.: $(-0.38, 0.23)$], and a disadvantage over nalidixic acid resistance in the nalidixic acid treatment [difference in growth = -0.75, 95% C.I.: $(-1.05, -0.45)$, Supplementary Information Section 2.2]. There is thus no evidence of a benefit to multi-resistance in single drug treatments, suggesting that multi-resistance did not spread due to positive selection.

## Growth of multi-resistant mutators and non-mutators

In addition to increasing the probability of resistance emerging, the elevated mutation rates of mutators may also affect the fitness achieved by multi-resistant lineages. We compared growth curves of multi-resistant clones arising at each mutator frequency, as well as with the multi-resistant lineages selected in the wild-type background by

fluctuation tests (used to parametrise the simulations). We obtained growth curves both in antibiotic-free medium and at the maximum concentration of the combination treatment (i.e. 0 mg/l and 20 mg/l) and summarised these by calculating area under the curve (AUC, Figure 3). We analysed the relationship between mutator frequency and AUC, allowing an additional effect of mutator frequency on the variance, using a Bayesian multivariate regression model (fully outlined in Supplementary Information Section 2.3). The growth of each strain at 0 mg/l and 20 mg/l was strongly positively correlated [$r = 0.73$, 95% C.I.: $(0.57, 0.84)$]. Expected AUC for each level of mutator frequency had overlapping 95% credible intervals (C.I.s). However, there was a lower average AUC for the 'low' frequency treatment. Higher mutator frequencies may have given populations access to resistance mutations that confer a larger fitness benefit[46]. The growth of multi-resistant mutators from the selection experiment did not differ considerably from those used to parametrise the simulations (i.e. had overlapping 95% C.I.s). However, variation in growth was higher for multi-resistant mutators from the selection experiment, potentially reflecting additional accumulated mutations[47,48]. Overall, the presence of mutators therefore had little effect on the typical fitness of multi-resistant lineages that emerged, but did potentially alter the variation in fitness across replicate populations.

## Stochastic simulations reveal evolutionary mechanisms of multi-resistance evolution

To gain insight into the drivers of resistance evolution, we used a stochastic population-dynamic simulation model. We simplified the underlying biology to produce a minimal model capable of predicting multi-resistance evolution. To do this, we included only a single allele for each resistance type, and excluded multi-drug resistance mechanisms, spontaneous double mutants, recombination, and mutations at non-resistance-conferring loci. Disallowing these types of variation permitted us to test hypotheses regarding the roles of selection and mutation supply on the emergence of multi-resistance. We use a stochastic approach because it allows the possibility novel mutants may become extinct while rare[49–51]. The populations initially comprise a sensitive wild-type strain, which we denote $S$. During growth, single mutations can occur, giving rise to rifampicin-resistant type $R$ and nalidixic acid resistant type $N$. Subsequently, $R$ and $N$ can each give rise to multi-resistant type $D$ (Figure 4). Mutations may occur in either the wild-type or mutator genetic background, resulting in a total of eight possible genotypes. Wild-type and mutator differ only in mutation rate. For each set

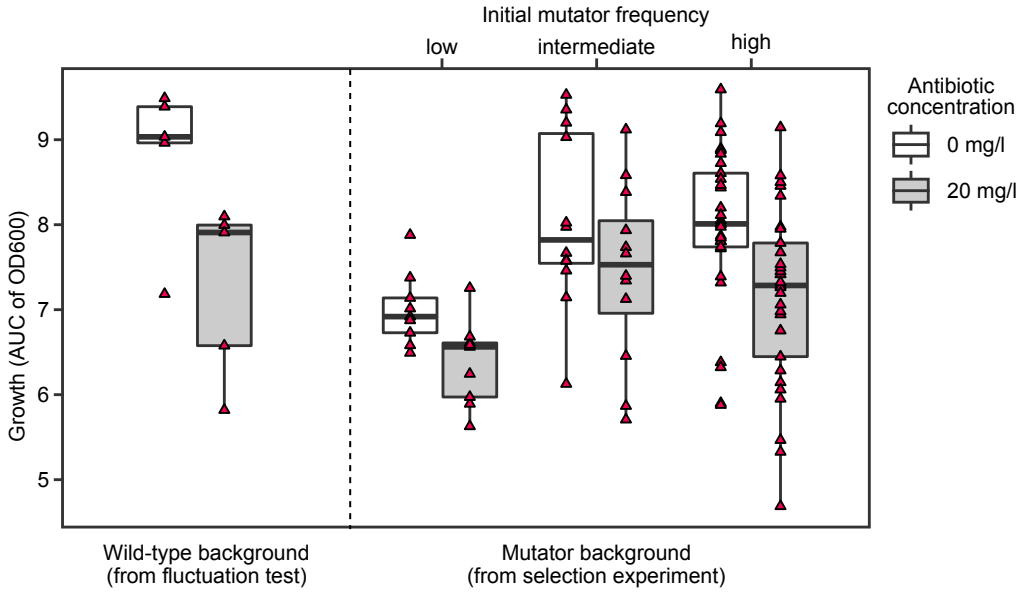


**Figure 3: Growth of multi-resistant mutants in the presence and absence of the antibiotic combination.** Growth curves were measured over 22 h at 0 mg/l and 20 mg/l (final concentration of the selection experiment). Box plots represent the median and interquartile ranges of area under the curve (AUC) of multi-resistant strains obtained via optical density at 600 nm. For the associated statistical analysis, see Supplementary Information Section 2.3.

of conditions, we simulated 1000 populations with a maximum population size of $5.71 \times 10^8$ bacteria, equivalent to the maximum density observed in the selection experiment. Other simulation parameters relating to growth were estimated empirically from growth curve data using strains that were derived from fluctuation tests (i.e. independent from the selection experiment, see Supplementary Information Section 1.6 for full details). Mutation rates of our wild-type and mutator strains were estimated by fluctuation assays previously performed by this group[19,52].

This minimal simulation captured the major features of the wet-lab experiments: mixed wild-type/mutator populations, increasing antibiotic concentrations, and periodic bottlenecks. Figure 5 shows the proportions of populations evolving resistance for 1000 replicate simulations (analogous to the experimental results shown in Figure 1). A Bayesian categorical model fitted to these simulations produced parameter estimates that closely matched those from the experimental data, demonstrating that the simulations quantitatively recapitulate the experiments (see Supplementary Information Section 2.4). In particular, we reproduce the main experimental findings that multi-resistance was

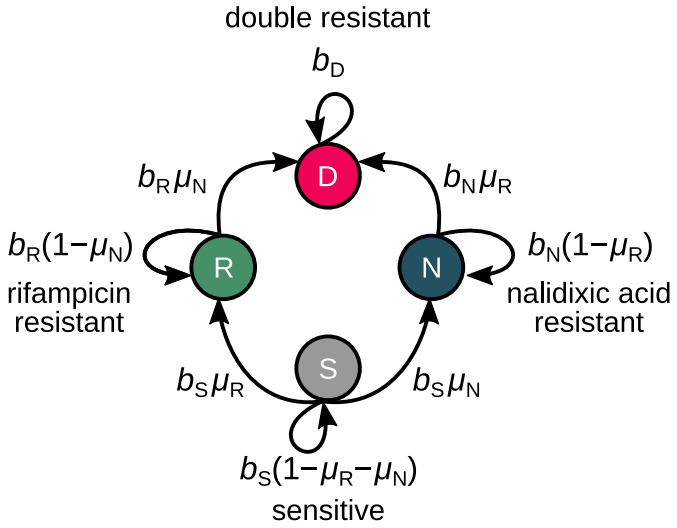


**Figure 4: Population dynamics simulation model of multi-resistance evolution.** The model describes individuals of strains $i \in \{S, R, N, D\}$ (sensitive, rifampicin resistant, nalidixic acid resistant, multi-resistant). Each individual of type $i$ produces an offspring with probability $b_i$ in each time step. A mutation to rifampicin resistance occurs with probability $\mu_R$, or nalidixic acid resistance with probability $\mu_N$. Simultaneous acquisition of both mutations in one reproduction event is not allowed in the model (i.e. $S$ cannot give rise to $D$, refer to Methods and Supplementary Information Section 1.1 for further details).

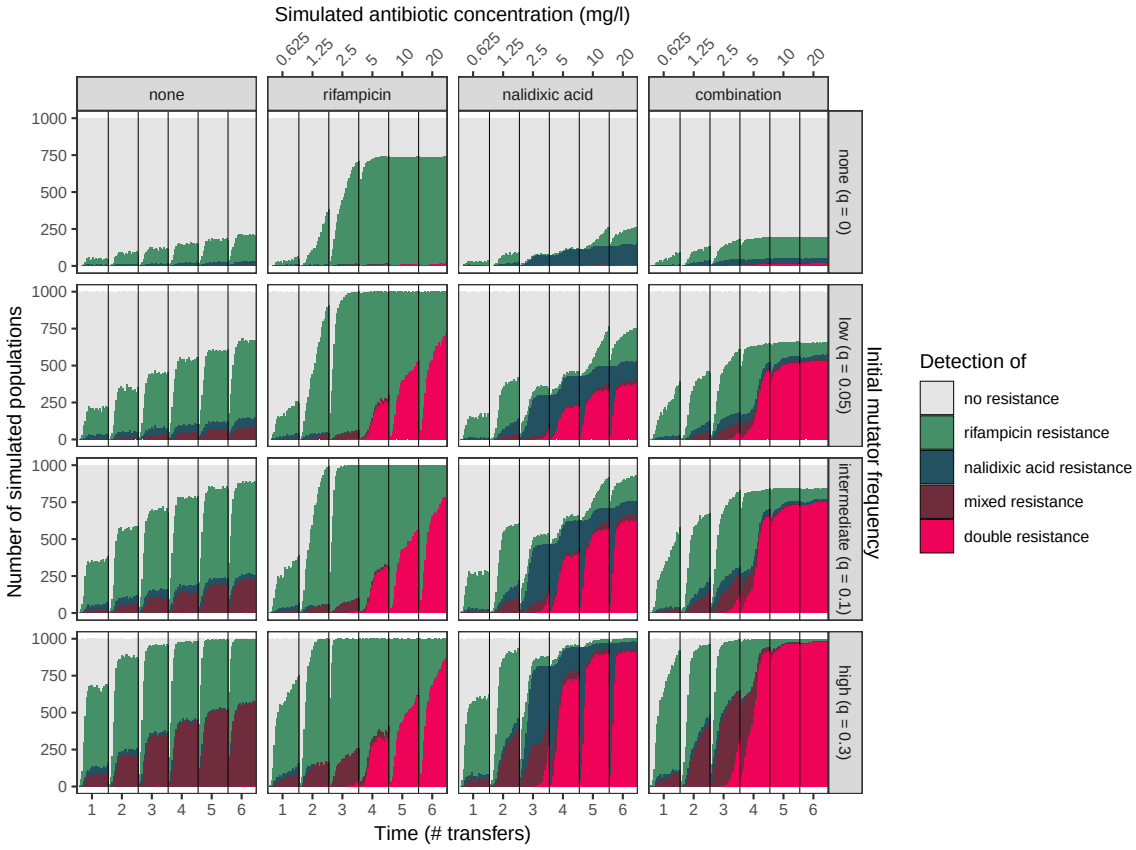


**Figure 5: Emergence of antibiotic resistance in stochastic simulations of resistance evolution.** Resistance state was determined from a random sample of 1/200 of the population (simulating the selective plating step in the experiments, see Methods). Parameters used in simulations are shown in Supplementary Figure 3. For the associated statistical analysis, see Supplementary Information Section 2.4.

constrained to populations treated with antibiotics, including single antibiotic treatments, and that the presence of mutators facilitated multi-resistance evolution. We found that a small fraction of purely wild-type populations evolved multi-resistance (between 0/1000 and 15/1000, depending on treatment); this is consistent with our experimental results ($< 1/60$ per treatment). As the simulation excludes the possibility of multi-resistance emerging through a single reproductive event, this suggests that multi-resistance can emerge without invoking multi-drug resistance mechanisms (e.g. efflux pumps), simultaneous acquisition of two resistance mutations, or recombination.

The simulations allow us to take a closer look at the population dynamics of resistance evolution within populations, starting from the sensitive type $S$ to the multi-resistant type $D$. This demonstrates that single resistance typically arises early on in the mutator genetic background, but remains at low frequency until it confers a fitness benefit over the sensitive type $S$. Once the single-resistant lineage begins to increase in frequency, a subsequent mutation producing the multi-resistant type $D$ can emerge and sweep to fixation. Figure 6 shows the most commonly observed evolutionary trajectory to multi-resistance, $S' \rightarrow R' \rightarrow D'$ (where prime refers to the mutator background); examples of other trajectories can be found in Supplementary Figure 8. In some cases, resistant types can be lost due to population bottlenecks (as is the case for $N$ in Figure 6 and $R$ and $D$ in Figure 8); these may re-emerge and go on to found a $D$ type lineage, or ultimately go extinct. $R$ and $N$ types can sometimes emerge and co-exit without going extinct, ultimately leading to two independent lineages resulting in $D$ type individuals. These results are summarised across all replicates for each antibiotic treatment in

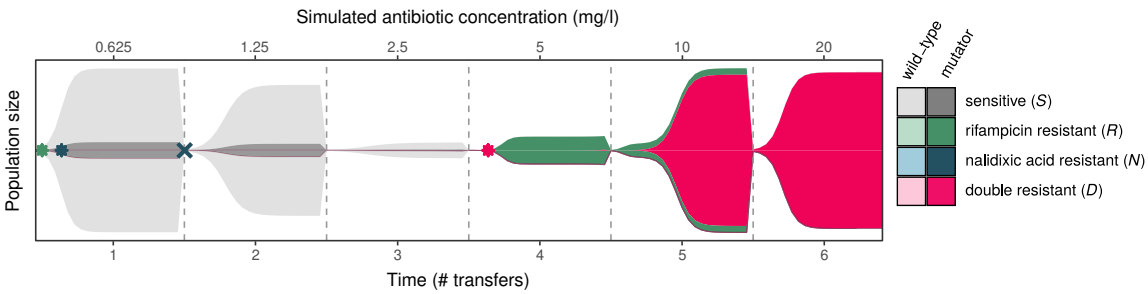


**Figure 6: Typical within-population progression from sensitive to multi-resistant in the combination treatment.** Multi-resistance most commonly emerged via $S' \rightarrow R' \rightarrow D'$ in the mutator genetic background (examples of other paths are shown in Supplementary Figure 8). Height of each area corresponds to the population size of each type. Example shown is a single replicate from the 'intermediate' initial mutator frequency ($u = 0.1$) treatment.

Figure 7, which shows the interquartile range (i.e. 25% and 75% quantiles of the number of bacteria of each type over time; see also Supplementary Figure 9). In all antibiotic treatments, we observe the spread of a single-resistant mutator to high frequency. However, while we observed the multi-resistant type $D$ in all antibiotic treatments, these tend to remain at low frequency in single-drug environments, only sweeping toward fixation in the combination environment where they have a fitness benefit.

We further tested the generality of our simulation model by removing aspects that were introduced to match the empirical growth curves of our strains. Our empirical growth curves did not resemble 'standard' logistic growth, which prompted us to fit a more accurate two-stage growth curve, reminiscent of diauxic growth (see Methods). Further, some estimated parameters were not indicative of a cost of resistance (Figure 3). We therefore additionally simulated a fitness cost for resistance, by reducing the growth rate and carrying capacities in environments where a particular resistance mutation would not

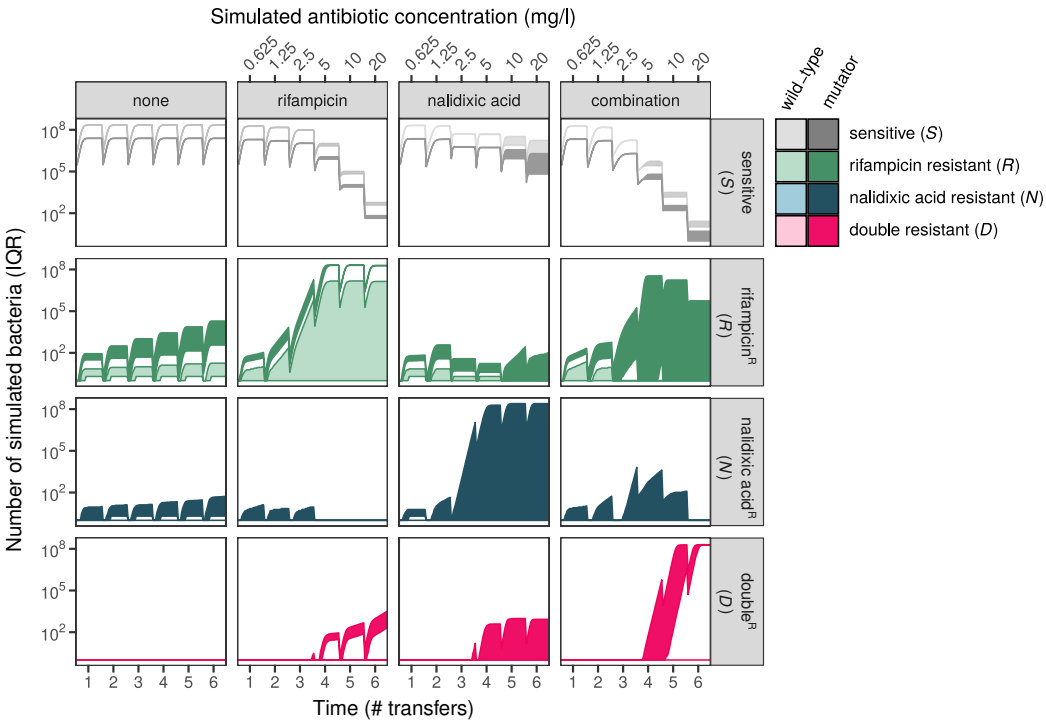


**Figure 7: Population dynamics of simulated resistance evolution.** Interquartile range (IQR) of the number of bacteria of each resistance type over time for the four simulated treatments (1000 replicate simulations). Results shown from the 'intermediate' initial mutator frequency ($u = 0.1$; other frequencies are shown in Supplementary Figure 9).

be expected to confer an advantage (i.e. environments without the relevant antibiotic). We also imposed a standard logistic growth model. We observed the same pattern of mutators facilitating multi-resistance under selection for either single-drug or multi-resistance (Supplementary Figure 11). Hence, our findings do not appear to be specific to our growth model or parameter estimates.

**Multi-resistance without mutators occurs in very large populations**

Our simulations have shown that mutators contribute to multi-resistance evolution because they increase the supply of resistance mutations, allowing independent resistance mutations to be acquired sequentially. However, mutational supply also depends on population size, which raises the possibility that multi-resistance may also evolve in large populations without mutators. We used our simulation model to determine how large a purely wild-type population would need to be to observe multi-resistance without mutators. Figure 8 shows the average proportion of each type of strain under different maximum population sizes after six days of simulated evolution. Multi-resistance reached appreciable frequencies for maximum population sizes between $5.71 \times 10^{10}$ (combination treatment) and $5.71 \times 10^{13}$ (no antibiotic treatment), which in all cases is orders of magnitude larger than the population size of $5.71 \times 10^{8}$ in our experimental conditions. Bacterial population sizes in infections are typically smaller than this range[53,54], although not always[55,56]. Commensal populations, however, can achieve population sizes in the range of $10^{10}$ or more[57,58], and $10^{13}$ bacteria is comparable in size to the entire human-associated microbiome[59]. Thus, multi-resistance may also be found without mutators in the largest microbial populations–although these populations also often contain mutators[27].

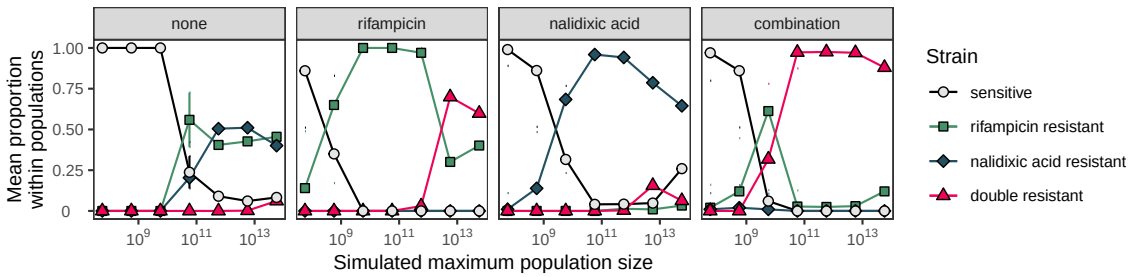


**Figure 8: Multi-resistance in the absence of mutators occurs in large populations.** Proportion of each strain type (±SD) found within simulated populations.

## Discussion

One of the main motivations for the use of combination antibiotic therapy is that it is thought to be more robust against resistance evolution because becoming multi-resistant through acquiring multiple independent resistance mechanisms is believed to be rare[10]. Contrary to this, here, we determined that multi-resistance can evolve in populations with a low-to-intermediate frequency of mutators (Figure 1). Strikingly, multi-resistance evolved not only under combination selection, where it was beneficial, but also under single-drug selection where it did not confer an overall advantage (Figure 2). Multi-resistant mutators that evolved through natural selection grew just as well as their counterparts selected in a wild-type genetic background (by sequential fluctuation tests, Figure 3); this indicates that is no inherent penalty (nor advantage) associated with evolving multi-resistance through an elevated mutation rate. Our results call in to question the main assumptions of combination therapy, that individuals possessing multiple independent resistance mechanisms should be rare, and that selection drives the emergence of multi-resistance. Given the prevalence of mutators in natural populations, including infections, our results suggest that combination antibiotic therapies may fail due to existing genetic variation for multi-resistance, even before being brought into use.

To discover the evolutionary mechanisms responsible for multi-resistance in mutator-containing populations, we used stochastic simulations parametrised with empirical estimates of growth parameters and mutation rates (Figure 4). We purposefully simplified the underlying biology to produce a minimal simulation model of multi-resistance evolution, which enabled us to test specific hypotheses. Our experimentally-evolved strains undoubtedly vary in various ways not captured by this minimal model (as suggested by the variation shown in Figure 3), but despite these simplifications, the match between experiments and simulation was remarkably close (Figures 1 and 5). The simulations revealed that mutators hitch-hike alongside single resistance mutations, and the resulting increase in frequency permitted a subsequent resistance mutation to occur in the same genetic background (Figure 6, consistent with previous work on mutators hitch-hiking with beneficial mutations[60–63]). As mutators were otherwise identical to wild-type in the simulations, increased mutation supply is a sufficient explanation for why multi-resistance evolves in mutator-containing populations. Multi-resistance also did not require multi-drug resistance mechanisms, cross resistance or spontaneous double mutations, as these routes to multi-resistance were excluded from the simulations. While different evolutionary mechanisms may play a role

in other settings, they need not be invoked here (e.g. differences in birth and death rates affecting the emergence of resistance[64], acquiring fitter resistance alleles through clonal interference[65], differential supply of compensatory mutations[66], genomic variation influencing MIC[67], or co-evolution between genome and resistance mechanisms[68]). Multi-resistance swept to fixation under conditions where it was beneficial, but remained present at a low detectable frequency in single-drug environments (Figure 7).

Our focus here was on genetically-encoded mutators bearing defects in DNA mismatch repair, as these are common in many types of clinical infection[23]. However, our results are likely to be equally relevant for other factors that affect mutation rates. These include, for instance, other genes not directly involved in mismatch repair[69,70], environmental factors (e.g. nutrients and temperature[71? –74]), antibiotics themselves (e.g. via stress-induced mutagenesis[75], radical-induced DNA damage[76,77], or inhibition of DNA synthesis and activation of error-prone polymerases[78–80]), and biotic interactions between organisms[19,69,81,82]. Theoretical models have also predicted that organisms with variable mutation rates may also have a higher probability of evolving multi-resistance[83]. In addition, our simulations suggest that multi-resistance may arise in large populations with wild-type mutation rates, albeit for population sizes that exceed those typical of infection (Figure 8). Other bacterial populations can, however, vary in size over several orders of magnitude[53–55], and our simulations suggest multi-resistance may arise without mutators in populations comparable to host-associated microbiomes[57–59]. Understanding these varied influences on the process of mutation will be critical in the discovery of methods for preventing resistance through altered mutation supply[84–86].

Many important clinical antibiotics are falling out of use due to the problem of mutational resistance, and there is hope that this can be reversed through antibiotic combination therapy. In addition to the two antibiotics considered here, resistance to various antibiotics can be achieved through spontaneous mutation, e.g. streptomycin (in *rpsL*), fosfomycin (in *murA*, *glpT*, or *uhpT*), cycloserine (in *cycA*), trimethoprim (in *folA* and its promoter), nitrofurantoin (in *nfsA* and *nfsB*) and some beta-lactams (in penicillin binding proteins). In addition, multi-drug resistance mechanisms can also be acquired through mutation (e.g. increased efflux and reduced uptake of drugs). What role mutators may play in the evolution of resistance to combination therapy in clinical contexts is therefore an important area for future research. Among clinical isolates, there is an established association between mutator phenotypes and multi-resistance[22], although this is thought to have arisen due to successive rounds of monotherapy. Whether multi-resistance can

arise during combination therapy, or even during monotherapy as suggested here, needs to be established. The observation of multi-resistance arising during combination therapies for HIV raises this as a possibility[42,87] (albeit at a mutation rate that exceeds even that of typical mutators[88]).

Aside from a higher propensity for antibiotic resistance, mutators present other challenges for infection management. Elevated mutation rates in general mean they are also better able to mitigate the fitness costs of antibiotic resistance through compensatory mutations[66]. Mutators can also more easily overcome non-antibiotic forms of bacterial control, such as vaccination[89] and phage therapy[90]. Unlike viruses, bacterial mutators are also unlikely to suffer from 'lethal mutagenesis'[91,92] or 'mutational meltdown'[93] (i.e. the accumulation of deleterious mutations leading to extinction) due to having large population sizes and mutation rates smaller than the error threshold[94]. Indeed, there is little evidence that mutators impair fitness over either short-term[95] or longer-term evolution[96] (except in the context of mutation accumulation experiments that keep population sizes artificially small[97]), and we found no evidence for it here (Figure 3). Together, this suggests that, once multi-resistant mutator lineages become established, they will be difficult to eradicate—whether through natural selection or through alternatives to antibiotics. This raises the possibility that screening for mutators, in addition to antibiotic susceptibility, could be valuable in clinical practice.

A vast number of combination treatments can be generated from existing antibiotics[98], which makes them an enticing approach for countering the rise of drug-resistant infections. While combinations treatments can indeed be useful for reducing resistance evolution[32,33], our results suggest that the potential for multi-resistance evolution, especially through the presence of mutators, needs to be considered in the design and application of such treatments. Moreover, as direct assessment of all possible combinations is likely an insurmountable task[98], the experimental and modelling approaches used here will also be useful in future as a framework for predicting which combinations will best suppress resistance evolution.

## Conflict of interest

The authors declare an absence of any conflicts of interest.

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## Author contributions

Conceived of experiment: DRG, CGK. Conducted experiments: CJ, DRG. Analysis of experimental data: EBC, DRG. Stochastic simulations: EBC, MS, DRG, TG. Initial draft of manuscript: DRG, EBC. Edited and approved final manuscript: all authors.

## Data availability

Data, scripts and source code are available on GitHub:
https://github.com/dannagifford/multi-resistance/

# Methods

## Strains and media

Selection experiments involved 'wild-type' *E. coli* str. K-12 substr. BW25113 [F-, Δ(*araD-araB*)567, Δ*lacZ*4787(::*rrnB*-3), λ-, *rph*-1, Δ(*rhaD-rhaB*)568, *hsdR*514][99], and a 'mutator' strain Δ*mutS* (as above, but with Δ*mutS*738::kan, indicating Δ*mutS* replacement with kanamycin resistance. The kanamycin resistance cassette has not previously been observed to affect resistance to the antibiotics we have considered here[19,52]). Both strains were obtained from Dharmacon, Horizon Discovery Group, UK. Relative to the published reference genome[100], whole genome resequencing of our isolates revealed no mutations in BW25113, and a single point mutation in Δ*mutS* (1,985,889 G>A, resulting in an amino acid substitution in *pgsA* A137V), which does not have a known association with resistance.

Routine culturing was performed in lysogeny broth [LB, 10 g/l tryptone (Fisher Scientific, UK), 5 g/l Bacto yeast extract (BD Biosciences, UK), 10 g/l NaCl (Fisher Scientific, UK)]. Selection experiments in the presence of antibiotic(s) were performed in Müller-Hinton broth (MH broth, 23 g/l, Sigma-Aldrich, UK). MH is a preferred medium for use with antibiotics[101]. Solid media were made by adding 12 g/l agar (BD Biosciences, UK) to either broth prior to autoclaving. Stock antibiotic solutions were prepared at 10 mg/ml. Rifampicin (Fisher Scientific, UK) was dissolved in methanol (Fisher Scientific, UK), and nalidixic acid (Fisher Scientific, UK) was dissolved in double distilled water, with 1N NaOH (Fisher Scientific, UK) added drop-wise until the antibiotic was solubilised. Strains were stored in LB with 40% glycerol at −80 °C.

## Selection experiment under single-drug and combination treatments

We used experimental evolution to determine the effect of mutators on multi-resistance evolution under single and combination antibiotic treatments. Populations were founded from a mixture of mutator and wild-type individuals. Independent overnight cultures of wild-type and mutator were first grown separately in 5 ml MH broth. Volumetric mixtures of the mutator and wild-type overnight cultures were made at ratios of 0/100% ('none'), 10/90% ('low'), 25%/75% ('intermediate'), and 50%/50% ('high'). These ratios were chosen to reflect mutator frequencies observed in host-associated populations[24–27]. The actual frequency of mutators in each mixed population (denoted $u$) was measured by plating serial dilutions of the populations on LB agar (total population count) and LB

with 100 mg/l kanamycin agar (mutator count) on 90 mm round Petri dishes (to account for differences between volumetric and actual proportions arising from both variation in density and experimental error; the actual mutator proportion was lower than the volumetric mixture). The initial mixtures were assayed for resistance to rifampicin or nalidixic acid by plating on MH agar supplemented with rifampicin (50 mg/l) or nalidixic acid (30 mg/l) on 90 mm round Petri dishes. We discarded 5 cultures for having detectable rifampicin resistance at the beginning of the experiment. No cultures had detectable nalidixic acid resistance. This was undertaken to observe the evolution of multi-resistance from an initially wild-type population, rather than single-drug resistance in an already resistant background. Resistance to these antibiotics arises due to mutations in *rpoB* for rifampicin[102], or in *gyrA* (Gram negative bacteria, such as *E. coli*) or *parC* (Gram positive bacteria) for nalidixic acid[103]. High-level resistance to either requires only a single base pair mutation in their target genes. Antibiotics in the parent classes of these antibiotics, rifamycins and fluoroquinolones, are often used in combination treatments (particularly against *M. tuberculosis*, where multi-resistance is rampant globally[104]).

We used a serial transfer protocol that exposed populations to increasing concentrations of antibiotics over a period of six days. The experiment was performed in 96-well microtitre plates (Nunc, Fisher Scientific, UK) in 200 µl volumes grown at 37 °C with 200 rpm shaking in an Innova 42R Incubator (Eppendorf, United Kingdom) for 22 h growth periods ('days'). Populations were initiated from the mixed cultures by diluting 1 µl of each into 200 µl of fresh culture using a 96-pin replicator (Boekel Scientific, Feasterville, PA, USA). At the end of each day, 1 µl of each population was pin replicated into 200 µl of fresh growth medium. If populations were to achieve the same size each day, a 1/200 dilution would imply $\log_2(200) \approx 7.64$ doublings per day.

Four antibiotic treatment regimes were used: no antibiotic, rifampicin only, nalidixic acid only, or rifampicin and nalidixic acid combined. Antibiotic concentrations were doubled each day over the course of six days (0.625, 1.25, 2.5, 5, 10, 20 mg/l of each individual antibiotic), where inhibition of the ancestral strain was achieved on day 5. The concentrations used in this dose escalation protocol are in the range of physiological concentrations achieved in blood during treatment with rifampicin (maximum serum concentration 5-18 mg/l[105]) and nalidixic acid (maximum plasma concentration 1.8-30 mg/l[35]). This type of dose escalation protocol is commonly used in resistance evolution experiments[3,106–108]. Under this approach, populations experience each concentration in sequence, hence resistance at higher concentrations is not independent of resistance at

lower concentrations (this differs conceptually from experiments that examine different fixed antibiotic concentrations, where different concentrations may select for different mutations[109,110]). This experimental protocol allows for a maximum of $\sim 46$ generations of evolution (i.e. six days $\times$ 7.64 doublings/day). However, in practice the number of generations achieved by non-resistant strains in the presence of antibiotic(s) is likely to be less, given their reduction in carrying capacity at higher antibiotic concentrations (see Supplementary Figure 3). We explore the potential for shorter and longer dose escalation protocols to influence multi-resistance evolution by using simulations (see Supplementary Information Section 1.9 and Supplementary Figure 10).

### Detection and analysis of resistance

Following each daily transfer, we assayed resistance by pin replicating 1 µl of overnight culture (equivalent to a random sample of 1/200th of the population) on MH agar without antibiotic, or with rifampicin (50 mg/l), nalidixic acid (30 mg/l), or rifampicin and nalidixic acid combined (50 mg/l and 30 mg/l, respectively) in 120 mm square Petri dishes. These concentrations are above the MIC, and resistance is indicative of mutations in the typical resistance genes for these antibiotics in *E. coli*, *rpoB* and *gyrA*, respectively. Other selective concentrations may yield different mutants[110]. We used the pin replicator method to allow a high-throughput assessment of the proportion of populations that contained individuals of each resistance type.

After overnight growth, populations were determined to be one of five 'resistance states': 'sensitive' if they only grew on non-selective plates, 'rifampicin resistant' or 'nalidixic acid resistant', if they grew on only one of the two single-drug plates, 'mixed resistant' if they grew on both single-drug selective plates but not combination selective plates, and 'multi-resistant' if they grew on combination selective plates (as well as single-drug selective plates). Note these outcomes refer to *establishment* of resistance, rather than *fixation*, i.e. the proportion of resistant individuals is $> 0$ and $\leq 1$. We used this approach because our focus was on estimating the proportion of populations that evolved multi-resistance, rather than the number of multi-resistant individuals that were present in any one population. After the sixth day, evolved populations that grew on the combination plates were grown overnight in LB medium, and then stored at −80 °C.

We analysed resistance using a Bayesian categorical model, implemented in the `brms` package[111,112] in R 3.5.3[113]. Resistance was treated as a categorical response variable

(categories defined as above), with antibiotic treatment ('no antibiotic', 'rifampicin', 'nalidixic acid', 'combination') and proportion of mutators ('none', 'low', 'medium', 'high') as categorical predictors. Row and column (i.e. position in the 96-well plate) were treated as random effects. We used Student's $t$ priors, with location $\mu$ and scale $\sigma$ estimated from a preliminary experiment, and degrees of freedom $\nu$ chosen to reflect uncertainty in the location, i.e. $t(\nu = 7, \mu = -5, \sigma = 2.5)$ for the intercept (i.e. the fitted value of the response when predictors take the reference level values 'no antibiotic' and 'zero' mutators). Priors for the parameters for the predictors were given as $t(\nu = 7, \mu = 0, \sigma = 2.5)$. We analysed both the 'main effects' of the predictors (i.e. the effect of a predictor on the response averaged across all levels of the other predictors), and their 'interaction effects' (i.e. the effect of a predictor being conditional on the value of another predictor). For an extended description of the analysis, see Supplementary Information Section 2.1.

### Growth parameters of single- and multi-resistant clones

To determine the effects of single and multi-resistance on growth parameters, we selected five nalidixic acid resistant and five rifampicin resistant clones arising from the wild-type BW25113 genetic background via fluctuation tests[114], using an established protocol[115]. Briefly, 1 ml LB cultures of *E. coli* K-12 BW25113 were grown overnight in 96-well deep-well plates. The entire volume of each culture was plated on MH agar supplemented with rifampicin (50 mg/l) or nalidixic acid (30 mg/l) in the wells of a 6-well plate (each well approximately 35 mm in diameter). These 6-well plates were incubated for 48 h. To select multi-resistant clones, we performed a second fluctuation test using resistant strains from the first, plating on the antibiotic to which they were not already resistant. Colonies were isolated from selective plates, grown overnight in LB medium, and then stored at −80 °C.

Growth curves were generated by measuring optical density (OD) at 600 nm every 30 min for 45 h using a BMG FLUOstar OMEGA with Microplate Stacker (BMG Labtech, Ortenberg, Germany, Supplementary Figure 1) The relationship between OD and colony forming units is shown in Supplementary Figure 5. Each clone was grown in duplicate at 37 °C under each of the antibiotic concentrations experienced during the selection experiments (i.e. 0.625, 1.25, 2.5, 5, 10, 20 mg/l each of rifampicin and/or nalidixic acid). Cultures were initiated by first growing clones overnight in 200 µl MH broth, then diluted 1/200 into a total volume of 200 µl MH broth containing one or both antibiotic(s). The

growth curves were used to estimate parameters for the stochastic simulation model, described below (and in full in Supplementary Information Section 1.6).

In addition to fitting growth curves, we also summarised these growth curves into a single metric, area under the curve (AUC), as the empirical growth curves did not follow a standard logistic shape (which we also account for in the simulations). We calculated AUC using the SummarizeGrowth function from the R package growthcurver[116]. AUC incorporates all of lag phase, growth rate, and density, and highly repeatable. To determine whether multi-resistance conferred a benefit under single-drug treatments, we fit a Bayesian multivariate linear regression model of AUC of different strains in the presence of each treatment over all concentrations. Student's $t$ priors were used for the intercept ($\nu = 7, \mu = 10, \sigma = 2.5$) and the other effects ($\nu = 7, \mu = 0, \sigma = 2.5$). Effects of single-drug and multi-resistance were compared with point hypothesis tests on the population-level effects from this model using 95% credible intervals (C.I.s). For an extended description of the analysis, see Supplementary Information Section 2.2.

Using the same protocol, growth curves for the multi-resistant clones that evolved during the selection experiment (all in the mutator genetic background) were measured in antibiotic-free medium, and in 20 mg/l of the combination treatment. The association between AUC, initial mutator frequency and treatment was analysed using a Bayesian multivariate regression model. Student's $t$ priors were used for the intercept ($\nu = 7, \mu = 10, \sigma = 2.5$) and the effect of mutator levels ($\nu = 7, \mu = 0, \sigma = 2.5$) for other estimated parameters (see Supplementary Information Section 2.3).

### Mutation rate estimates and the probability of spontaneous double mutants

Mutation rates to rifampicin resistance ($\mu_R = 6.7 \times 10^{-9}$ per cell division) and nalidixic acid resistance ($\mu_N = 7.4 \times 10^{-10}$ per cell division), were obtained for the these strains in a previous publication[52], as was the mutator effect of $\Delta mutS$ (80-fold increase relative to wild type)[19]. Assuming mutations occur independently, we estimate the probability of both rifampicin and nalidixic acid resistance mutations occurring simultaneously during the same replication event (i.e. a 'spontaneous double mutant') for the wild-type as the product of their mutation rates to resistance[1,20], i.e. $\mu_R \mu_N = 5.0 \times 10^{-18}$, and for mutators as $\mu_R \mu_N \times 80^2 = 3.2 \times 10^{-14}$. We can use these probabilities to obtain a rough estimate for the probability of observing a spontaneous double mutant by simulating a fluctuation test using `rflan()` from the R package 'flan'[117]. We performed $10^6$ simulations using the

same parameters as the selection experiment (i.e. 60 independent populations, days 1–4 permitting wild-type growth, maximum population size of $5.71 \times 10^8$). For populations comprising only wild-type individuals, no spontaneous double mutants were observed in $10^6$ simulations. For populations comprising solely mutators, a spontaneous double mutant was observed in 4210 out of $10^6$ simulations. Hence, the probability of observing a spontaneous double mutant is very low.

### Stochastic population dynamics model

We numerically simulated resistance evolution using a stochastic population dynamic model, described in Supplementary Information Section 1. Briefly, the model describes four strain types $i \in \{S, R, N, D\}$, where $S$ is the sensitive ancestor, $R$ is rifampicin resistant, $N$ is nalidixic acid resistant, and $D$ is multi-resistant (Figure 4). Simulated populations were initiated with $5.71 \times 10^6$ individuals of type $S$; this is our estimate of the starting population size in the experiments obtained by serial dilution plating. Of the initial population of Type $S$ individuals, a fraction $u$ were designated mutators ($u = 0, 0.05, 0.1$, or $0.3$, approximating the observed frequencies in the 'none', 'low', 'medium' and 'high' mutator treatments) and the remaining $1 - u$ were designated wild-type (i.e. non-mutators). Bacterial growth was modelled as a Yule-Furry process, but in addition allowing the reproduction rate to depend on population density. As the experiment involved a serial transfer protocol, population bottlenecks were also simulated.

At the start of the simulation, there were no Type $R$, $N$, or $D$ individuals. Type $R$ and $N$ individuals must initially arise by mutation in reproduction events of Type $S$ individuals. Once single mutants of Types $R$ or $N$ exist in the population, they may produce further such individuals by reproduction. In reproduction events of individuals of Types $R$ or $N$, a subsequent mutation can occur, producing Type $D$ individuals. Type $D$ individuals may also reproduce, but we do not include further mutations in the model. We write $\mu_R$ for the probability with which an offspring acquires resistance to rifampicin by mutation, and $\mu_N$ for the probability that the offspring acquires resistance to nalidixic acid. We exclude the possibility that both resistance mutations can be newly acquired in the same reproduction event (i.e. no multi-drug resistance mechanisms, and no double-mutation events, which we have shown in the preceding section to be rare).

Parameters used in the simulations were estimated from growth curves using strains that

were derived in an experiment independent from the selection experiments (see Supplementary Information Section 1.6). For each resistance type, we used five clones derived from the wild-type BW25113 background via fluctuation tests. We used wild-type-derived clones due to inconsistency in the growth curves obtained using the $\Delta mutS$ mutator strain (due to adaptation occurring during the assay, a difficulty encountered when working with mutators[118]). The shape of the growth curves did not follow a standard logistic shape, but is reminiscent of diauxic growth (Supplementary Information Figure 1). We therefore fit a two-stage growth curve involving two growth rates ($r_i^{(1)}$ and $r_i^{(2)}$) and two carrying capacities ($k_i^{(1)}$ and $k_i^{(2)}$) for each strain $i$ (Supplementary Figures 2 and 3). Growth rates and carrying capacities both contribute to fitness, and hence to determining which strains will increase in frequency under given conditions. Growth curves for each clone were fitted in MATLAB2016a, using a custom script (see 'Data availability' statement). As we aimed to produce a model that captures the main experimental results with as few parameters as possible, we considered only one allele of each resistance type. We thus took the average parameter value over the five independent clones.

As described in the preceding section, mutation rates used were estimated by fluctuation test in a previous study using these specific strains[19]. Other parameter values were set to match the experimental procedure (initial frequency of mutators, dilution, duration of experiment). The relationship between optical density and colony forming units was used to convert between OD units and numbers of bacteria (Supplementary Information Figure 5).

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