Mutators drive evolution of multi-resistance to antibiotics

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Abstract

Combination therapy aims to prevent growth of organisms not resistant to all component drugs, making it an obvious strategy for countering the global rise of multi-drug resistance. However, success relies on preventing resistance from arising to all component drugs before full inhibition is reached during treatment. Here, we investigated whether bacterial populations can overcome combination therapy by evolving 'multi-resistance', i.e. independent resistance mutations to multiple drugs, during single-drug and combination antibiotic treatment. Using both experimental evolution and *in silico* stochastic simulations, we studied resistance evolution in a common laboratory strain of bacteria (*Escherichia coli* K-12 BW25113). Populations were exposed to either single-drug or combination treatments involving rifampicin and nalidixic acid, with concentrations increasing through time. For wild-type populations, multi-resistance was not detected in any of the experimental populations, and simulations predict its evolution should be rare. However, populations comprising mixtures of wild-type and 'mutator' strains were readily capable of evolving multi-resistance. Increasing the initial frequency of mutators resulted in a higher proportion of populations evolving multi-resistance. Experiments and simulations produced the same qualitative-and in many cases, quantitative-insights about the association between resistance, mutators and antibiotic treatment. In particular, both approaches demonstrated that multi-resistance can arise through sequential acquisition of independent resistance mutations, without a need to invoke multi-drug resistance mechanisms. Crucially, we found multi-resistance evolved even when not directly favoured by natural selection, i.e. under single-drug treatments. Simulations revealed this resulted from elevated mutation supply caused by genetic hitch-hiking of the mutator allele on single-drug resistant backgrounds. Our results suggest that combination therapy does not necessarily prevent sequential acquisition of multiple drug resistances via spontaneous mutation when mutators are present. Indeed both combination and single-drug treatments actively promoted multi-resistance, meaning that combination therapy will not be a panacea for the antibiotic resistance crisis.

Keywords antibiotic resistance, evolution, mutation rate, ramping selection, stochastic simulations, individual-based model, Markov chains

Introduction

The global threat of antimicrobial resistance is spurring research into the effective use of existing drugs to prevent further resistance evolution. There has been sustained interest in the use of combination therapy for preventing resistance evolution in viral, bacterial and fungal infections, and in cancer (e.g. Devita *et al.*, 1975; Bonhoeffer *et al.*, 1997; Livermore, 2005; Baym *et al.*, 2016). Combination therapy uses multiple drugs concurrently to treat a single infection, in contrast to other strategies involving more than one drug, e.g. 'mixing' (assigning different antibiotics to different patients), and 'cycling', (using two antibiotics alternately). Modelling has shown that combination therapy provides an advantage of combination therapy over these other strategies (Tepekule *et al.*, 2017). Further, combination therapy has proved successful in a number of clinical applications, particularly in the management of HIV, which has led to a substantial decline in resistant infections (Lopez and Banerji, 2016; Antiretroviral Therapy Cohort Collaboration, 2017). There is optimism that wider deployment could reduce evolution of resistance in bacterial infections, where the burden of resistance is increasing rapidly (Palmer and Kishony, 2013). Considerable progress has been made in characterising how the interactions between antibiotics used combinations affect their ability clear infections and prevent the spread of resistance mutations (e.g. Hegreness *et al.*, 2008; Torella *et al.*, 2010; Pena-Miller *et al.*, 2013; Baym *et al.*, 2016; Barbosa *et al.*, 2018).

The ability of combination therapy to block resistance evolution relies on preventing preventing the establishment of a clonal lineage with 'multi-resistance', i.e. resistance to more than one drug via multiple independent mutations (which we distinguish from 'multi-drug resistance' that arises through a single mutation, e.g. in multi-drug efflux pump regulation). In bacteria, simultaneous acquisition of multi-resistance should be exceedingly rare. For independent resistance mutations, the rate of simulatenous multi-resistance is the product of mutation rates to resistance for each drug (with per-drug estimates approximately 10^{-8} to 10^{-10} per genome replication, Krašovec *et al.*, 2017). Multi-resistance is therefore more likely to occur via sequentially acquiring resistance to each component drug (Bonhoeffer *et al.*, 1997). Although combination therapy aims to prevent sequential acquisition, failure to achieve inhibitory concentrations of all component drugs has been shown to permit sequential multi-resistance evolution in multiple systems (Foo and Michor, 2009; Moreno-Gamez *et al.*, 2015; Feder *et al.*, 2017). Antibiotics do not reach fully inhibitory concentrations

immediately upon treatment, particularly when administered orally, which increases the propensity for resistance (Felton *et al.*, 2013). For combination treatments, this may allow organisms to survive long enough to acquire sequential resistance mutations.

A particular complication of treating bacterial infections is heterogenetiy in mutation rates within populations. Notably, 'mutators', i.e. organisms with mutation rates 10–1000-fold higher than wild-type, often arise due to defects in DNA mismatch repair (reviewed in Marinus, 2012). Bacterial infections often comprise a mixture of wild-type and mutator organisms at varying frequencies, e.g. in urinary tract infections caused by *Escherichia coli* (4.2%–62.5% mutators, Couce *et al.*, 2016), and in cystic fibrosis-associated infections caused by *Pseudomonas aeruginosa* (~53% mutators, Oliver *et al.*, 2000; Maciá *et al.*, 2005). Mutators present at these frequencies have been shown to increase the rate of adaptation of populations (e.g. Taddei *et al.*, 1997; Giraud *et al.*, 2001; Desai and Fisher, 2011; Raynes *et al.*, 2018). By reducing the waiting time for mutations to occur, mutators could exacerbate the problem of sub-inhibitory concentrations facilitating multi-resistance evolution. Although there is an established link between mutator alleles and multi-resistance (Chopra *et al.*, 2003), whether the presence of mutators can enable populations to evolve multi-resistance during the course of a combination treatment is yet unknown.

Using a combination of experimental evolution and stochastic simulations, here we tested whether mutators facilitate multi-resistance evolution during antibiotic treatment. We measured the probability of drug resistance evolution in populations of bacteria varying in the initial proportion of mutators exposed to no-drug, single-drug, or combination treatments that increase in concentration over time. Experiments were performed with a laboratory strain of bacteria (*E. coli* K-12 BW25113) and a mismatch repair-deficient derivative (Δ*mutS*), using the antibiotics rifampicin and nalidixic acid. Simulations were parameterised with fitness and mutation rates measured independently from the experiments. We found that mutators played a critical role in multi-resistance evolution both experiments and simulations. In the absence of mutators, no multi-resistance was observed experimentally, and simulations suggest it should be rare even when beneficial. Strikingly, when mutators were present, we observed multi-resistance evolution in both of the single-drug treatments and in the combination treatment. Different evolutionary mechanisms explain multi-resistance in these treatments. In the single-antibiotic treatments, multi-resistance arose due to genetic hitch-hiking of the mutator allele along with single-drug resistance, allowing increased mutational supply (de Visser, 2002). In

contrast, in the combination treatment, multi-resistance arose due to positive selection, sweeping to high frequency as the concentration of both antibiotics increased. There was no association between the mutator allele and fitness deficits. Given the prevalence of mutators, multi-resistance evolution may present a real challenge to the wider deployment of combination therapy against bacterial infections.

Methods

Strains and media

Selection experiments involved 'wild-type' *E. coli* strain K-12 substrain BW25113 [F-, Δ(*araD-araB*)567, Δ*lacZ*4787(::*rrnB*-3), *λ*-, *rph*-1, Δ(*rhaD-rhaB*)568, *hsdR*514], and a Δ*mutS* 'mutator' strain (as above, but with Δ*mutS*738::kan, indicating *mutS* replacement with a kanamycin resistance cassette). Δ*mutS* was originally constructed as part of the Keio collection (Baba *et al.*, 2006), and both strains were obtained from Dharmacon, Horizon Discovery Group, UK. Strains were grown in Müller-Hinton broth (MH broth, 23 g/l, Sigma-Aldrich, UK) or 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)], as indicated. Solid medium was 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 cultures were made at ratios of 0%, 10%, 25%, and 50% mutator culture. The actual proportions were measured by plating serial dilutions of the populations on

LB agar (total population count) and LB with 100 mg/l kanamycin agar (mutator count). 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); any culture found to have resistance already present was discarded.

We used a serial transfer protocol, exposing populations to increasing concentrations of antibiotics over a period of 6 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 6 days (0.625, 1.25, 2.5, 5, 10, 20 mg/l of each individual antibiotic). Population density was measured each day by optical density at 600 nm using a BMG POLARstar OPTIMA (BMG Labtech, Ortenberg, Germany).

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). These concentrations are indicative of resistance in the typical resistance genes for these antibiotics in *E. coli*, *rpoB* and *gyrA*, respectively. Populations were assigned one of five categories: 'sensitive' if they only grew on non-selective plates, 'rifampicin resistant' or 'nalidixic acid resistant', respectively, if they grew on one of the two single-drug plates, 'mixed resistant' if they grew on both single-drug selective plates but not combination selective plates, and 'double resistant' (i.e. multi-resistant to two antibiotics, the simplest form of multi-resistance) if they grew on combination selective plates. Note these outcomes refer to *detection* of resistance, rather than *fixation*, i.e. the frequency of resistant individuals is > 0 and ≤ 1. Evolved populations that grew on the combination plates at the end of the

sixth day 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 (Bürkner, 2017, 2018) in R 3.5.3 (R Core Team, 2019). Resistance was treated as a categorical response variable (categories defined 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 μ and scale σ estimated from a preliminary experiment, and degrees of freedom ν chosen to reflect uncertainty in the location, i.e. $t(\nu = 7, \mu = -5, \sigma = 2.5)$ for the intercept and $t(\nu = 7, \mu = 0, \sigma = 2.5)$ for other estimated parameters (see Figure S1 and Model M1 in the supplementary information). From the model, we discuss main effects (i.e. the effect of a predictor on the response averaged across all levels of the other predictors), and interaction effects (i.e. the effect of a predictor being conditional on the value of another predictor).

Fitness of single- and double-resistant clones

To determine fitness effects of single and double resistance, we selected five nalidixic acid resistant and five rifampicin resistant clones arising from the wild-type BW25113 genetic background via fluctuation tests (Luria and Delbrück, 1943), using the protocol developed by Krašovec *et al.* (2014). 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) and incubated for 48 h. To select double-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 of single- and double-resistant clones were generated by measuring optical density at 600 nm every 30 min for 45 h using a BMG FLUOstar OMEGA with Microplate Stacker (BMG Labtech, Ortenberg, Germany). Clones were grown in duplicate at 37 °C under each of the antibiotic concentrations experienced during the selection experiments. 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). We assayed six concentrations (corresponding to the conditions described in the selection experiment): 0.625, 1.25, 2.5, 5, 10, 20 mg/l each of rifampicin and/or nalidixic acid. Wells exhibiting resistance emergence during the assay, as indicated by a replicate growth curve diverging significantly from the mean, were excluded from analysis. These data were used to determine parameters for the simulation model (as described in the supplementary information). As a proxy for fitness, we also used this growth curve data to estimate area under the curve (AUC) for the first 25 h of growth using the SummarizeGrowth function from the R package growthcurver (Sprouffske and Wagner, 2016). To determine whether double resistance conferred a benefit under single-drug treatments, we fit a Bayesian multivariate 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 $t(\nu = 7, \mu = 0, \sigma = 2.5)$ (see Figure S2 and Model M2 in the supplementary information). Effects of single-drug and double resistance were compared with point hypothesis tests on the population-level effects from this model using 95% credible intervals (C.I.s).

Using the same protocol, growth curves for the double-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 Model M3 in the supplementary information).

Stochastic population dynamics model

We numerically simulated resistance evolution using a stochastic population dynamic model. The model describes four strains of type $i \in \{S, R, N, D\}$, where S is the sensitive ancestor, R is rifampicin resistant, N is nalidixic acid resistant, and D is double resistant (Figure 1). We denote the number of strain-i individuals in the population by n_i. Simulated populations were initiated with 5.71×10^6 individuals; this is our estimate of the starting population size in the experiments obtained by serial dilution plating. The initial population consisted of a fraction $1 - q$ of wild-type individuals, and a fraction q of mutators, for $q \in \{0, 0.05, 0.1, 0.3\}$. Parameter values were estimated empirically (growth rates and carrying capacities, given in Tables S7 and S8), taken from the literature

(Krašovec *et al.*, 2018, mutation rate), or were set to match the experimental procedure (initial frequency of mutators, dilution, duration of experiment). Mean growth rates and carrying capacities were estimated from kinetic growth curve data from five single- and double-resistant clones in the wild-type *E. coli* K-12 BW25113 genetic background using MATLAB 2016a (see Figures S4 and S5 and supplementary information).

Population growth is described as follows. Considering first population growth without mutation, for each time step, each of the n_i individuals of strain i produces one offspring with probability $b_i = \exp\{\Delta t r_i (1 - n_T/k_i)\} - 1$ for $n_T/k_i \leq 1$, where r_i is the net growth rate (per hour) for strain i, and k_i is the carrying capacity. At stationary phase (i.e. $n_T/k_i \geq 1$), $b_i = 0$ to ensure that n_i remains constant. We have written $n_T = \sum_i n_i$ for the total number of individuals in the population. The quantity Δt is the time step of our simulations (we use $\Delta t = 0.25$ h, although smaller time steps produced qualitatively similar results; further details can be found in the supplementary information). In each time step, the number of offspring of strain i is binomially distributed, Binomial(n_i, b_i). Population growth periods were 22 h, equivalent to 'days' of the experiment. At the end of each day, a dilution occurs in which the number of individuals of type i transferred to the next day is binomially distributed, Binomial$(n_i, 1/200)$. In the supplementary information we show that, for growth without mutations, the mean number of individuals of each strain follows the deterministic Leslie-Gower competition model (Leslie and Gower, 1958, a discrete-time model conceptually similar to the Lotka-Volterra competition model in continuous time, Volterra 1926; Lotka 1932).

Type R, N, and D individuals can also arise by mutation from new individuals that are produced. We write μ_R for the probability with which an offspring acquires resistance to rifampicin by mutation, and μ_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. The number of individuals of type i arising by mutation in any one time step is therefore Binomial$(n_S, b_S \mu_i)$ for $i \in \{R, N\}$. For $i = D$ the number of individuals generated through mutation is the sum of two binomial random numbers, Binomial$(n_R, b_R \mu_N)$ and Binomial$(n_N, b_N \mu_R)$. We use the resistance mutation rates $\mu_R = 6.7 \times 10^{-9}$ and $\mu_N = 7.4 \times 10^{-10}$ mutations per cell division, obtained from the rich-media data in Krašovec *et al.* (2018).

Simulations were written in C++ (source code available in the supplementary information). To compare simulations with the experimental results, we drew a random

sample equivalent to 1/200th of all individuals from the simulated population whenever data was taken from the simulation. Detection of type i was recorded for a given population if a draw from Binomial(n_T, $n_i/(200 \cdot n_T)$) was at least 1. This is equivalent to the resistance detection assay for the experimental results.

Results

Mutators facilitate double resistance evolution under single-drug and combination treatments

We subjected populations of bacteria with different initial mutator frequencies to increasing concentrations of either single or combination antibiotic treatments. Figure 2 shows the number of populations with detectable resistance under different antibiotic treatments (as detected by selective plating, though optical density revealed a similar trend, Figure S3). Single-drug resistance was observed at some point during the experiment for all initial mutator frequencies and treatments. When antibiotics were present, the number of resistant populations generally increased through time, excepting populations without mutators. However, the proportion of populations with resistance was roughly U-shaped in the absence of antibiotics for all mutator frequencies (left column of Figure 2). In contrast, multi-resistance was only observed when mutators were present, and only in the presence of antibiotics. Where it was detected, multi-resistance was preceded by single-drug resistance in every population except for one, providing evidence for sequential, rather than simultaneous, acquisition of multi-resistance.

The relationship between resistance at the final time point, mutators and antibiotic treatment was determined using a Bayesian categorical mixed-effects model (see Model M1 in the supplementary information). The fit of a full model incorporating both main effects and interactions was only marginally better than a main effects-only model, hence we present parameter estimates from the latter (given in full in Table S1 in the supplementary information). The main effect of mutators was positive for all resistance types (i.e. having 95% C.I.s for the posterior greater than zero), with the exception of 'low' mutators and nalidixic acid resistance. While the mean effects of mutators increased with increasing mutator frequencies (low < intermediate < high, generally), the 95% C.I.s of their posteriors overlapped. Single antibiotic 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 double resistance evolving.

Fitness estimates suggest no selection for double resistance in single-antibiotic treatments

The occurrence of double-resistant strains in single-antibiotic treatments is surprising. We therefore used fitness assays to test whether there could be any positive selection for double resistant strains in the presence of single antibiotics. To determine which treatments should result in positive selection for resistance, we assayed fitness of sensitive, single- and double-resistant clones under all conditions experienced during experimental evolution. Fitness 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. For the combination treatment, there was a clear advantage of double resistance over single-drug resistance for concentrations of 1.25 mg/l and above (Figure 3). These concentrations correspond to time periods where double resistance begins to be detected in the experiments. In contrast, there was no clear fitness advantage of double resistance over rifampicin resistance in the rifampicin treatment [difference in effect = -0.07, 95% C.I.: $(-0.38, 0.23)$], and a disadvantage over nalidixic acid resistance in the nalidixic acid treatment [difference in effect = -0.75, 95% C.I.: $(-1.05, -0.45)$]. This provides no evidence of a benefit to double resistance in single drug treatments, suggesting that double resistance did not spread due to positive selection.

Mutators did not impair fitness of multi-resistant strains

Although we have established that mutators may increase the probability of observing multi-resistance, a potential consequence of elevated mutation rates is the accumulation of deleterious genetic variation. This may impair the fitness of multi-resistant clones arising in mutators, suggesting they may be rapidly outcompeted by fitter clones if antibiotics are removed. To determine the effect of initial mutator frequency on the fitness of evolved multi-resistant clones, we compared growth curves of clones that evolved in mutator genetic background during combination treatment against clones in the

wild-type background. Recall that we used successive fluctuation tests to isolate double resistant mutants in the wild-type background. This was performed so that we could estimate fitness effects of resistance mutations with a minimum number of mutations at other loci in the genome. We analysed the association between multi-resistant fitness and mutator frequencies using a Bayesian multivariate regression model (see Model M3 in the supplementary information). A model incorporating initial mutator frequency did not improve fit over an intercept-only model, suggesting the initial frequency of mutators did not have a large overall influence on fitness in either environment. However, the 'low' initial frequency of mutators was associated with lower fitness in the absence of antibiotics, but we found no further evidence that the fitness of multi-resistant strains arising in the mutator genetic background was lower than in the wild-type background in either environment (Figure 4). Further, fitness of individual clones assayed in each environment was positively correlated [residual $r = 0.69$, 95% C.I.: $(0.56, 0.79)$], providing no evidence of a trade-off between fitness in the presence and absence of the combination treatment. This suggests that there is no drawback to multi-resistance arising in mutator genetic backgrounds, as might be expected if elevated mutation rates allow deleterious mutations to accumulate (Söderberg and Berg, 2011).

Insight into evolutionary mechanisms using stochastic simulations

If double resistance does not confer a benefit under single-drug treatments, different mechanisms must explain its prevalence under single-drug and combination treatments. To gain a mechanistic insight into the drivers of double resistance evolution in these treatments, we simulated resistance evolution using a stochastic population-dynamics model involving diauxic logistic growth subject to competition among strains, mutation, and periodic population dilutions. Model parameters were estimated from experimental data on mutation rates of wild-type and mutator strains (Krašovec *et al.*, 2017), and growth rates of sensitive and resistant strains measured at each concentration of antibiotic(s) (Figure 3). Figure 5 shows the proportions of populations evolving resistance for 1000 replicate simulations (analogous to the experimental results shown in Figure 2). A Bayesian categorical model fitted to simulations produced parameter estimates that closely matched those from the experimental data, demonstrating that the simulations quantitatively recapitulate the experiments (Figure S7 and Model M4).

In particular, we reproduce the main experimental findings that double resistance was

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

Figure 6 shows summary statistics for simulated population dynamics for the 'high' ($p = 0.3$) initial mutator frequency , i.e. numbers of sensitive, single-drug resistant, and multi-resistant bacteria (examples of individual populations are shown in Figure S7, and summary statistics for other initial mutator frequencies in Figure S8). In single-drug treated populations, mutators swept to high frequency due to genetic linkage with single-drug resistance (i.e. in the same genome). Multi-resistance then arose subsequently in a population now comprising mostly single-resistant organisms, although reached only low relative frequency due to the absence of selection for multi-resistance. In contrast, in combination-treated populations, multi-resistance swept to fixation without fixation of single-drug resistance, due to direct selection for multi-resistance. Increasing the frequency of mutators decreased variability in the number of multi-resistant bacteria present at the end of the simulation (Figure S8). This reveals different evolutionary mechanisms responsible for multi-resistance in single-drug and combination treatments: genetic hitch-hiking of mutator alleles under single-drug treatments, and direct selection for sequentially-evolved multi-resistance under combination treatment.

Discussion

One of the primary motivations for the use of combination antibiotic therapy is reducing the probability of resistance evolution (Pletz *et al.*, 2017). Here, we study the evolution of multi-resistance evolution to two antibiotics under single-drug and combination treatments. Using experiments and simulations, we demonstrated that mutators facilitate the evolution of double resistance in bacterial populations comprising both wild-type and mutator individuals (Figure 2). Double resistance occurred both in single-drug treatments and the combination treatment, despite conferring no fitness advantage in single-drug

treatments (Figure 3). There was no fitness deficit associated with double resistance having arisen in a mutator genetic background (Figure 4). We simulated resistance evolution to explore the mechanisms responsible for double resistance (Figure 5). Simulations showed that double resistance can arise via sequential acquisition of resistance to each antibiotic, rather than simultaneously. Double resistance arose due to genetic hitch-hiking of the mutator allele in single-drug treatments, and direct selection in the combination treatment (Figure 6). Taken together, these results suggest that combination therapy may not be a fool-proof strategy for preventing antibiotic resistance evolution, given the prevalence of mutators in bacterial infections.

We showed that mutators facilitated multi-resistance evolution under single-drug and combination treatments in both simulation and experiment (Figures 2 and 5). Our aim with simulations was to predict the probability of multi-resistance evolution with a model involving as few parameters as possible. This allowed us to demonstrate which biological phenomena are necessary to predict multi-resistance evolution with reasonable accuracy. Specifically, our model considers only eight possible genotypes (i.e. four resistance states in wild-type and mutator genetic backgrounds), whereas in reality, there are many possible resistance alleles. Further, the model does not include adaptive or compensatory mutations at other loci. Finally, we specifically excluded double-mutation events and multi-drug resistance mutations to demonstrate that multi-resistance could evolve in their absence. The good quantitative match between experiment and simulation (Figure S9) suggests that these aspects are not required for the model to predict single-drug resistance and multi-resistance. However, there were two differences between model and experiment that may have arisen from these simplifications. In the 'no antibiotic' treatment, we found that the relationship between rifampicin resistance and time was U-shaped in the experiment and monotonic in the simulation (first columns of Figures 2 and 5). This difference may be explained by selective sweeps of adaptive mutations replacing rifampicin resistance mutations, which are generally costly in rich medium in *E. coli* K-12 (Reynolds, 2000) and also in other species (Hughes and Brandis, 2013). We also detected more mixed resistance in the experiment, primarily in the nalidixic acid treatment (third columns of Figures 2, 5 and S9). The simulation could have underestimated mixed resistance if there were additional fitness costs of double resistance that were not captured by our model (e.g. differences in lag time). However, even with inexact quantitative matches for these two resistance states in these two environments, the key qualitative predictions hold. This demonstrates that insight into complex

evolutionary processes can be gained from relatively uncomplicated stochastic models.

Whether multi-resistance evolution evolves during the clinical application of antibiotic combination therapy, and whether mutators influence this process, is yet unknown. Resistance to HIV combination therapy emerging in clinical trials (6%-15% of patients Sax *et al.*, 2011) suggests this possibility, although the mutation rate of HIV is several orders of magnitude larger than that of bacterial mutators (Cuevas *et al.*, 2015). Sequencing and resistance profiling of clinical bacterial isolates has demonstrated a link between multi-resistance and elevated mutation rates. In particular, genetic defects in DNA mismatch repair are associated with multi-resistance in multiple bacterial species, e.g. chronic *P. aeruginosa* infections (Ferroni *et al.*, 2009), clinical isolates of *Acinetobacter baumannii* (Komp Lindgren *et al.*, 2015), and in blood and urinary tract isolates of *E. coli* (Miller *et al.*, 2004; Baquero *et al.*, 2004; Labat *et al.*, 2005). Genetic hitch-hiking of mutator alleles with resistance presents a challenge for infection management, as such infections increase their evolutionary potential to develop resistance against future antibiotics and other forms of bacterial control, such as vaccination (Bayliss *et al.*, 2008) and phage therapy (Pal *et al.*, 2007). Further, there is little support for mutators suffering from decreased fitness, both over short time scales studied here (Figure 4), and over longer-term evolution (Couce *et al.*, 2017), and may even compensate better for the costs of resistance (Perron *et al.*, 2010). This suggests that once multi-resistance in a mutator lineage becomes established, it would be exceptionally difficult to eliminate. Collectively, this raises the question of whether screening for mutators, in addition to antibiotic susceptibility, would be valuable in clinical practice.

Although here we have focused on genetically-encoded mutators, environmental factors influencing mutation rates may also contribute to multi-resistance evolution. Mutation rate appears to be a phenotypically plastic trait across all domains of life (Krašovec *et al.*, 2017), though the precise mechanisms through which this operates has yet to be established. Recent work has shown a link between mutation rate and efflux gene expression (El Meouche and Dunlop, 2018). Environmental stress and mutagens can also transiently elevate mutation rates, e.g. via stress-induced mutagenesis (Petrosino *et al.*, 2009), radical-induced DNA damage (Kohanski *et al.*, 2010), or inhibition of DNA synthesis and activation of error-prone polymerases (Gillespie *et al.*, 2005; Henderson-Begg *et al.*, 2006; Thi *et al.*, 2011). This may explain the high rate of multi-resistance in *Mycobacterium tuberculosis*, which is thought to have a relatively low mutation rate (McGrath *et al.*, 2013). Beyond increasing average mutation rates,

increasing the variability has also been shown to increase the probability of multi-resistance (Alexander *et al.*, 2017). Understanding the mechanistic underpinnings of mutation rates, and the relationship with resistance, will open the possibility for drugs that inhibit resistance evolution through suppressing mutations (Ragheb *et al.*, 2018).

Our results suggest that indiscriminately pairing antibiotics could have drastic consequences for the propensity for populations to evolve multi-resistance. Although there are many potential combinations that can be generated by randomly pairing current antibiotics (Wood, 2016), these findings indicate a need for a rational approach to designing combination therapies. Antibiotics in the same classes as those used in our experiments, the rifamycins and fluoroquinolones, are often components of combinations, particularly against *M. tuberculosis*, where multiple antibiotic resistance is rampant globally (Manson *et al.*, 2017). Resistance to antibiotics in these classes arises readily due to mutations in their 'resistance determining regions', in *rpoB* for the rifamycins (Goldstein, 2014), or in *gyrA* (Gram negative bacteria) or *parC* (Gram positive bacteria) for the fluoroquinolones (Redgrave *et al.*, 2014). High-level resistance to either requires only a single base pair mutation in their target genes, in contrast to other antibiotics that require multiple mutations (e.g. trimethoprim, Palmer *et al.*, 2015). This particular combination of classes is therefore likely to perform particularly poorly. However, beyond testing a specific combination treatment, our stochastic simulation model provides a framework for evaluating new combinations based on experimental measurements of fitness and mutation rates. Future investigation into the rational design of combination therapy is needed to identify which efforts, such as minimising mutational target sizes, or matching pharmacokinetics to minimise periods of sub-inhibitory concentrations, will help to prevent the evolution of multi-resistance during combination treatment.

Conflict of interest

The authors declare an absence of any conflicts of interest.

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Author contributions

Conceptualization (DRG, CGK), Data Curation (CJ, DRG), Formal Analysis (DRG, EBC, TG, CGK), Funding Acquisition (DRG, EBC, TG, CGK), Investigation (CJ, EBC), Methodology (EBC, DRG), Project Administration (DRG, TG), Resources (CGK), Software (EBC), Supervision (DRG, TG, CGK), Validation (DRG, EBC, TG), Visualization (DG, EBC), Writing–Original Draft Preparation (DG, EBC), Writing–Review & Editing (All authors).

Data archiving

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

Figures

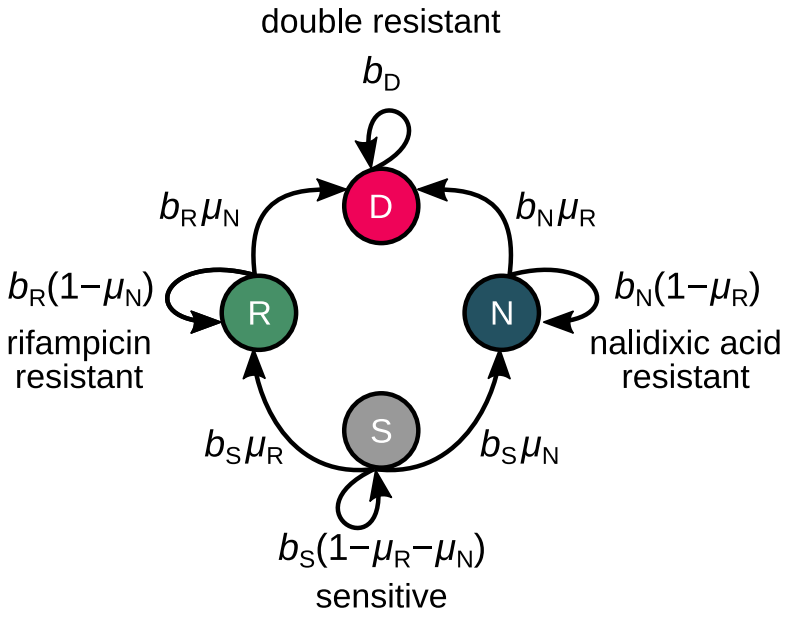

Figure 1: Population dynamics model of multi-resistance evolution. The model describes individuals of strains $i \in \{S, R, N, D\}$ (sensitive, rifampicin resistant, nalidixic acid resistant, double resistant). Each individual of type i produces an offspring with probability b_i in each time step. Of those, a rifampicin resistance mutation occurs with probability μ_R (if not already rifampicin resistant), or a nalidixic acid resistance mutation occurs with probability μ_N (if not already nalidixic acid resistant). Simultaneous acquisition of both mutations in one reproduction event is not considered in the model (i.e. S cannot give rise to D).

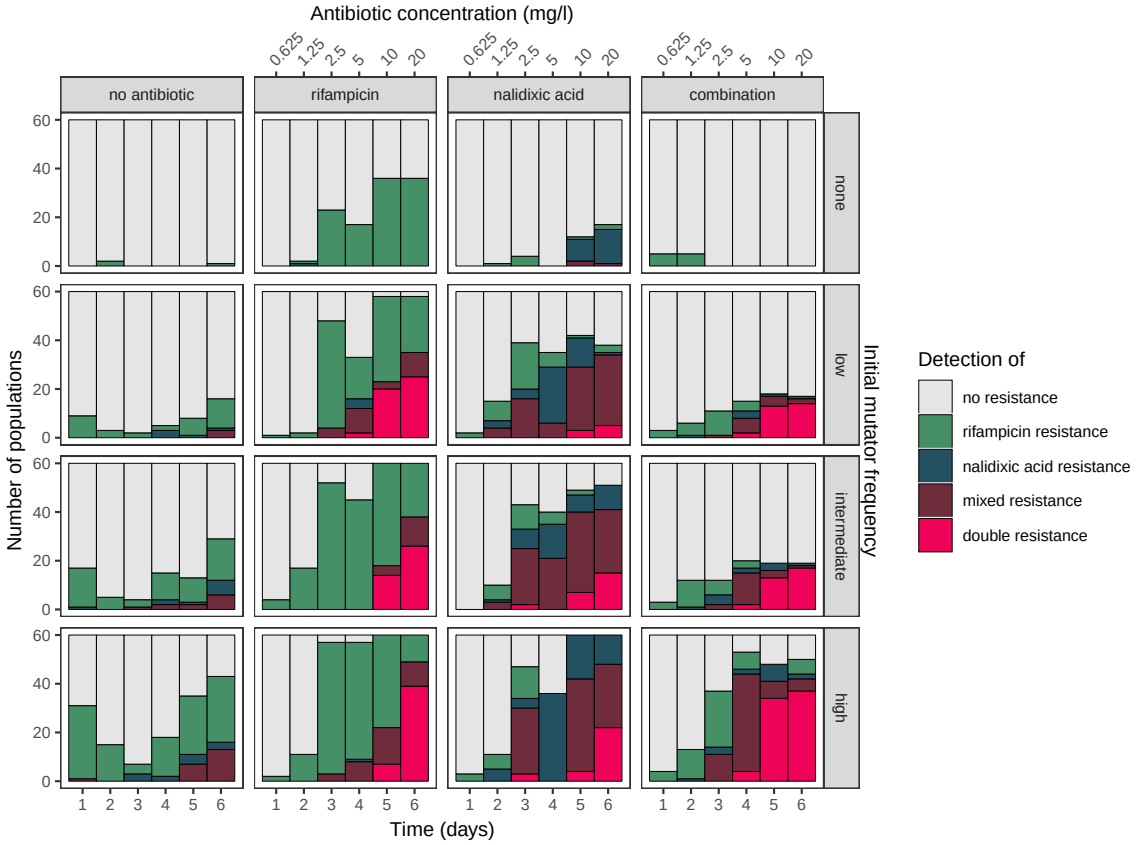

Figure 2: Emergence of antibiotic resistance in the presence and absence of selection for resistance in populations with and without mutators. Multi-resistance emerged when mutators were present (rows) under both mono- and combination antibiotic therapy (middle and right columns, respectively), but not in the absence of antibiotics (left column). Stacked bars show numbers of populations where each category of resistance was detected by selective plating. 'Rifampicin resistance' and 'nalidixic acid resistance' indicate growth on either rifampicin or nalidixic acid medium, respectively, but not on both nor in combination. 'Mixed resistance' indicates growth on both rifampicin and nalidixic acid medium separately but not in combination. 'Double resistance' indicates growth on both antibiotics separately and also in combination. See Model M1 in the supplementary information.

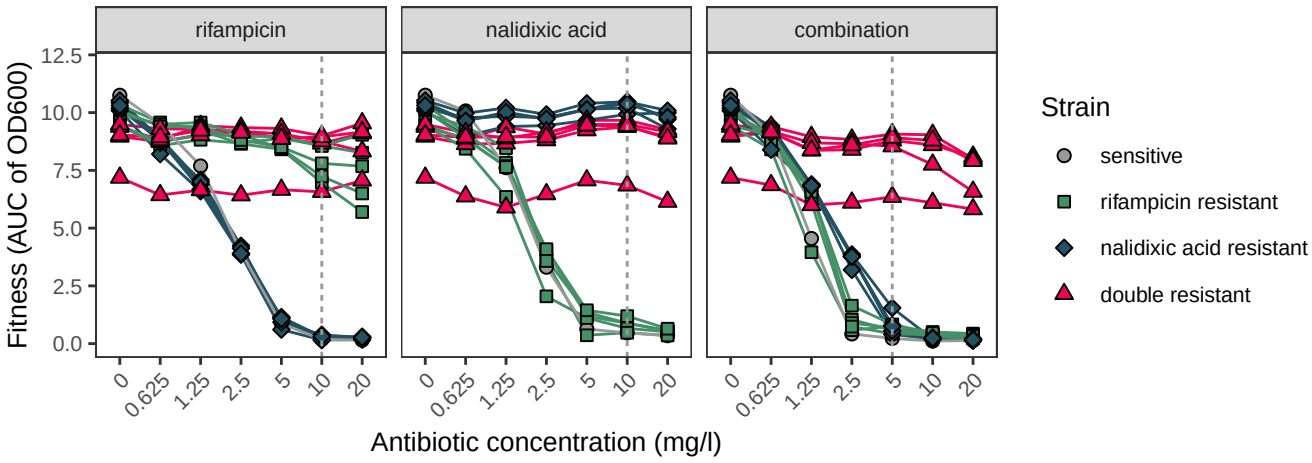

Figure 3: Fitness of sensitive, single-, and double-drug resistant strains at antibiotic concentrations experienced during the resistance selection regime (AUC of OD600: area under curve of growth curves measuring optical density at 600 nm over time). Resistant strains ($n = 5$) were isolated in the wild-type *E. coli* K-12 BW25113 genetic background by fluctuation test(s). Vertical line indicates minimum inhibitory concentration of the wild-type. Error bars are ± 1 standard error across a minimum of two replicates, but are smaller than plotting symbols. See Model M2 in the supplementary information.

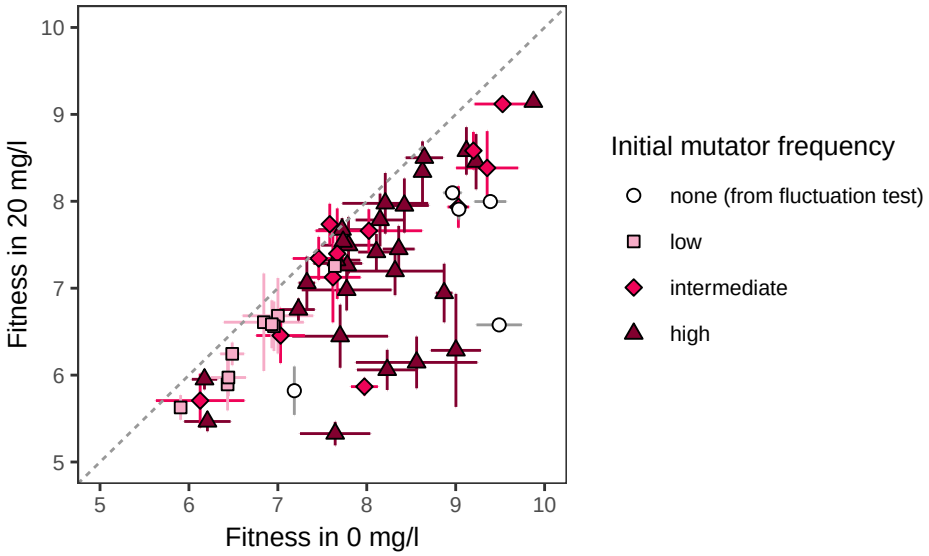

Figure 4: Fitness of double-resistant clones in 0 mg/l and 20 mg/l of the combination treatment (defined as area under curve of growth curves measuring optical density at 600 nm over time). Double resistant strains arising in the mutator genetic background during selection (pink polygons) were not less fit than double mutants selected during successive fluctuation tests in the wild-type *E. coli* K-12 BW25113 genetic background (white circles). Dashed diagonal line indicates equal fitness in both environments. See Model M3 in the supplementary information.

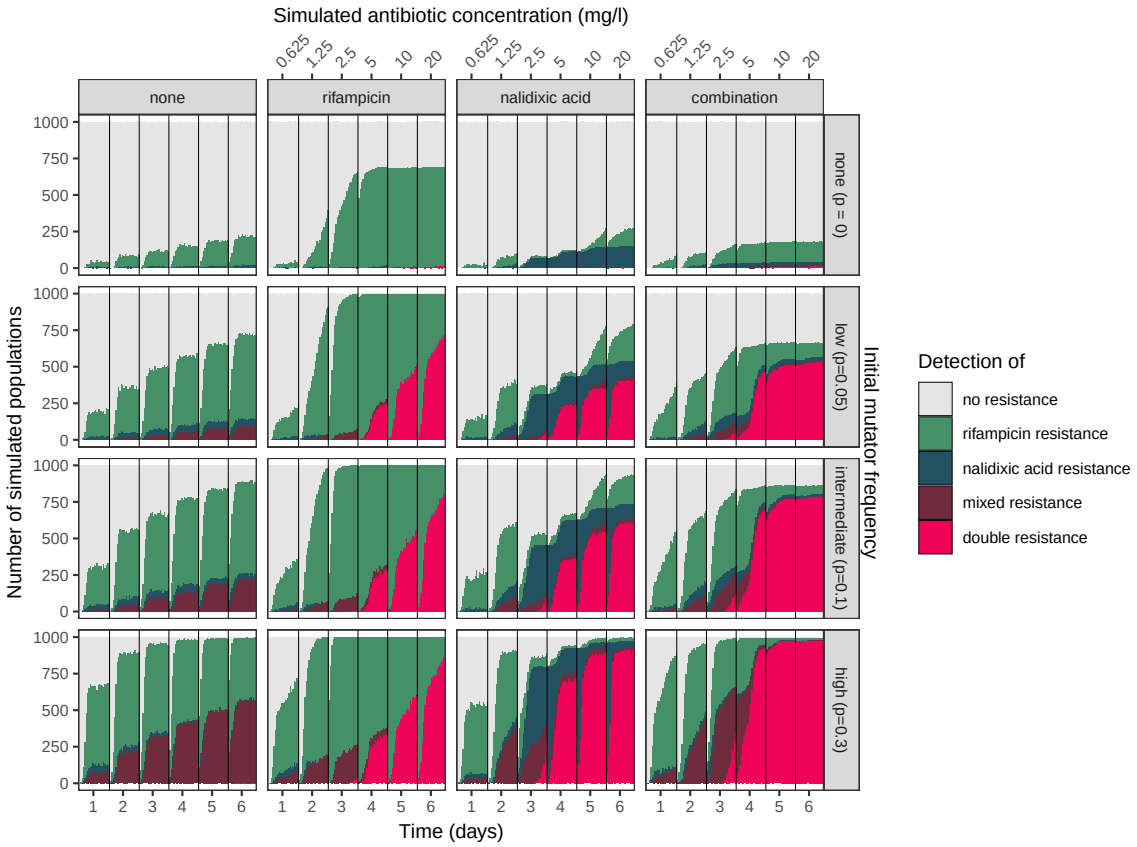

Figure 5: Emergence of antibiotic resistance in stochastic simulations of resistance evolution. Stacked bars show the number of populations with individuals of the types shown in Figure 1. 'Detection of' refers to the types of individuals present in a random sample of 1/200 of the population (simulating selective plating, see Methods), during each time step of the simulation. 'No resistance' indicates only type S was sampled (no R, N, or D). 'Rifampicin resistance' indicates at least one R (no N or D, any S), and 'nalidixic acid resistance' at least one N (no R or D, any S). 'Mixed resistance' indicates at least one each of R and N (no D, any S). 'Double resistance' indicates sampling at least one D (any S, R, or N). See Model M4 in the supplementary information.

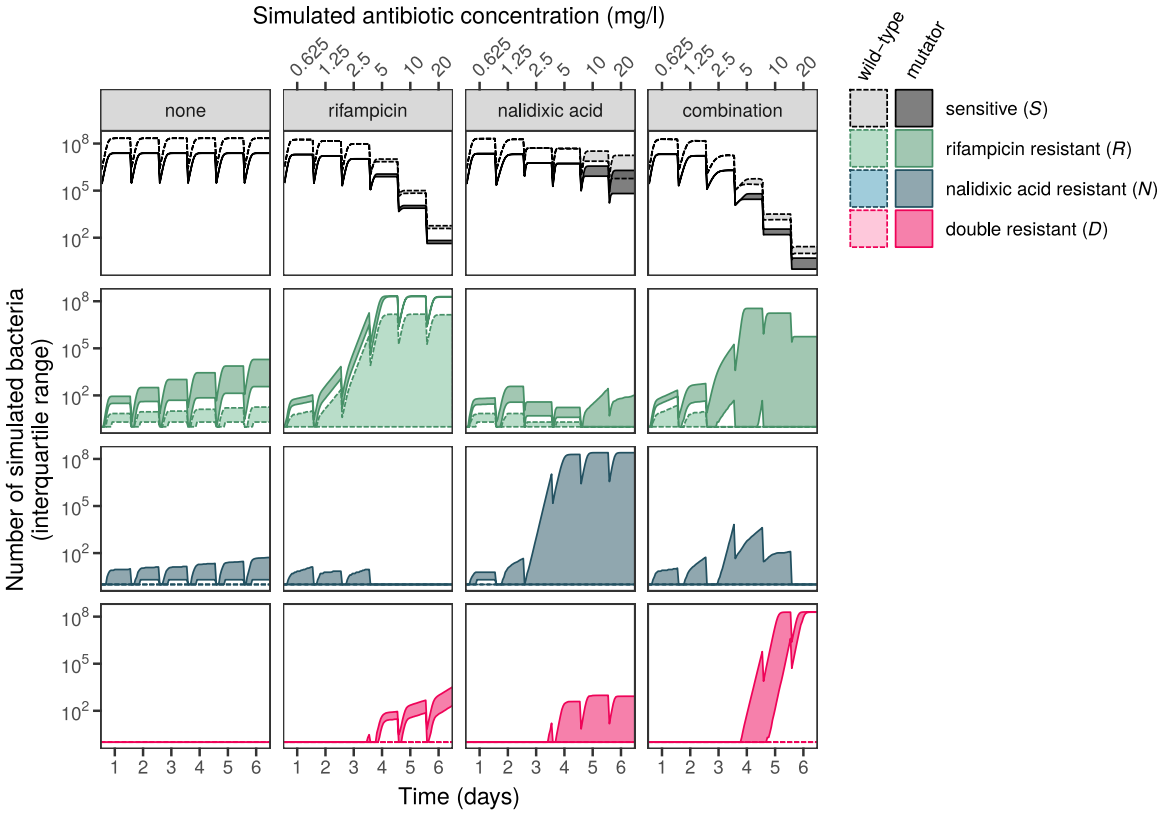

Figure 6: Population dynamics of simulated resistance evolution. Panels show the interquartile range (25% and 75% quantiles) of the number of bacteria of each resistance type (colours) for four treatments (columns) from 1000 replicate stochastic simulations. Results from the 'intermediate' ($p = 0.1$) initial mutator frequency are shown (other frequencies are shown in Figure S8).

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