Title: Strong Selective Agents Determine Resistance Evolution in a Multidrug Therapeutic Regime

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Abstract: Multidrug regimes have been considered to constrain selection for resistance compared to monotherapy. However, drug resistance trajectories are influenced by a wide range of conditions which can cause opposing outcomes. Here we employed an *in vitro* model system to investigate differences in resistance dynamics between mono-, combination and alternating regimes. Across regimes involving three drugs and phage, selection for resistance was decreased in multidrug regimes compared to monotherapy. Surprisingly, across regimes, two out of the four agents used to impose selection had a dominant effect on the overall outcome. Resistance to these agents either caused cross-resistance or obscured the phenotypic effect of other resistance mutations. This indicates that under multidrug therapeutic regimes, a small number of drugs dominate the emergence of antimicrobial resistance.

One Sentence Summary: Resistance evolution in multidrug environments is driven by a small subset of selective agents through pleiotropy and fitness costs.

Microbial populations harbor and rapidly generate tremendous genetic variability. This allows selection to act on them dramatically over brief time scales (days to weeks). Indeed, rapid microbial evolution has been frequently reported in response to a variety of selection pressures, including anthropogenic factors such as pharmaceuticals and agrochemicals (*1*). Features contributing to rapid evolution in microbial systems include large population sizes, short generation times, and the capacity to horizontally traffic genetic elements between taxa in communities. Identifying the factors determining evolutionary trajectories in microbial populations, and quantifying their impact, is of broad general interest as well as being tantamount to the efficacy of pathogen control efforts. These include tackling the increasing prevalence of antimicrobial resistant microbial infections in human healthcare and animal agriculture. The antimicrobial resistance crisis, rooted in large-scale anthropogenic antimicrobial use over the past decades, is considered to be one of the greatest challenges faced by humanity today (*2*).

Even the simplest form of adaptation can have numerous off-target consequences on the fitness and phenotype of an organism. For instance, a typical consequence of antimicrobial resistance mutations is impaired growth in the absence of the antimicrobial compared to susceptible cells, denoted as the fitness cost of resistance (*3*). This is because antimicrobials frequently target key cellular structures (e.g. ribosome or RNA polymerase) or functions (e.g. cell wall synthesis) important for fitness, and resistance mutations modify these very same structures or functions. However, the fitness cost of resistance is a coarse measure reflecting widespread changes in bacterial physiology and gene regulation. These changes can affect various phenotypic features in bacteria in addition to resistance to the selective antimicrobial. Among such pleiotropic phenotypes, those most commonly studied are susceptibilities to other antimicrobials, with cases of cross-resistance and collateral susceptibility often reported (*4*). From a broader perspective, the physiological consequences of antimicrobial resistance mutations also alter the fitness landscape for further mutations. In line with this, epistatic interactions between antimicrobial resistance mutations and other mutations are commonplace (*5, 6*). Pleiotropy and epistasis could also cause historical contingency in antimicrobial resistance evolution. A recent study found support for this, showing that resistance mutations to one antimicrobial compound from past exposure could influence the evolution of resistance to a second antimicrobial in the future (*7*).

Antimicrobial resistance factors experience positive selection when the fitness of resistant cells exceeds the fitness of susceptible cells in a population. The fitness advantage must surpass the physiological fitness-impairing consequences of resistance mutations as well as a threshold level of noise where the selection coefficient is too low to cause directional selection for resistance (*8*). This so-called minimum selective concentration (MSC) has been reported to occur *in vitro* at a range of 1/230 to 1/4 of the minimum inhibitory concentration (MIC) preventing the growth of susceptible cells (*9*). The MSC depends on the antimicrobial compound and type of resistance mutation. Moreover, in realistic habitats and community settings, microbes are often likely to simultaneously experience several abiotic and biotic selection pressures. Additional selective pressures can either obscure or exacerbate selection by the antimicrobial compound. For bacteria, these include bacteriophages *i.e.* viruses infecting bacteria (hereafter, phages), which are highly prevalent in natural habitats. Phages are also themselves increasingly employed as antimicrobial agents alone or in combination with antimicrobial compounds (*10*). Phages and antimicrobial compounds typically have different modes of action and targets of resistance mutations (*11*). This may constrain antimicrobial resistance evolution in phage-antimicrobial combination therapy. Alternatively, as bacterial cells experience double the stress in combination therapy, this may also aggravate selection for resistance mutations (*12*).

It remains unclear how the factors discussed above contribute to differences in resistance trajectories between three principal regimes: monotherapy, combination therapy and alternating therapy. Choosing the best regime for mitigating resistance *(i.e.* evolutionarily informed therapy) is critical for extending the shelf-life of existing antimicrobials. Combination therapy has been proposed to be superior to monotherapy by imposing more stringent conditions for resistance evolution at both ecological (rapid population crash shortening time window for *de novo* resistance mutations to occur) and genomic levels (engagement of multiple resistance targets which may also involve fitness trade-offs) (*13*). Drawbacks include enhanced selection for multidrug resistance when resistances are genomically coupled, conditions permit the stepwise accumulation of multiple resistance mutations, or multidrug resistance is encoded on mobile genetic elements such as conjugative plasmids. Alternating therapies can be divided into antimicrobial cycling at the level of the population and alternating (*i.e.* sequential therapy) at the level of the individual (*14-17*). Alternating therapies have also been shown to be predominantly superior to monotherapy. Similar to combination therapy, alternating therapy may involve fitness trade-offs between resistances, constraining selection. Limited time windows of selection can also decrease directional selection for individual resistances, especially if accompanied by fitness trade-offs causing reversal of the previous resistance in the presence of the next antimicrobial. Risks associated with alternating protocols include a high likelihood for pleiotropic effects from individual resistances, including cross-resistance to other antimicrobials. The selective conditions may also be less stringent compared to combination therapy allowing the stepwise accumulation of multiple resistance mutations.

Here we set out to investigate the differences in antimicrobial resistance dynamics between mono-, combination and alternating therapy using a serial passage experiment with *Escherichia coli*. We used three antimicrobials with different modes of action (nalidixic acid, rifampicin and spectinomycin) as well as the virulent phage T4. For the antimicrobials, we imposed subinhibitory selection (½ × MIC) clearly beyond the highest estimate for minimum selective concentration (up to ¼ × MIC) to allow resistance dynamics to be modulated by a wider range of factors compared to high-level selection (causing immediate fixation of resistance alleles present in standing genetic variation). We conducted the experiment for 48 days, including in our alternating therapy setup all 24 possible permutations of four 12-day epochs, consisting of three antimicrobial epochs and one antimicrobial-free epoch (Fig. 1). We performed the experiment at high replication (16 replicates for each unique treatment combination), amounting to a total of approximately 900 serially passaged populations. To investigate resistance evolution, we cross-phenotyped populations and clones isolated from the experimental end-point for resistance to each agent, as well as whole-genome sequencing over 200 clones representing divergent resistance outcomes.

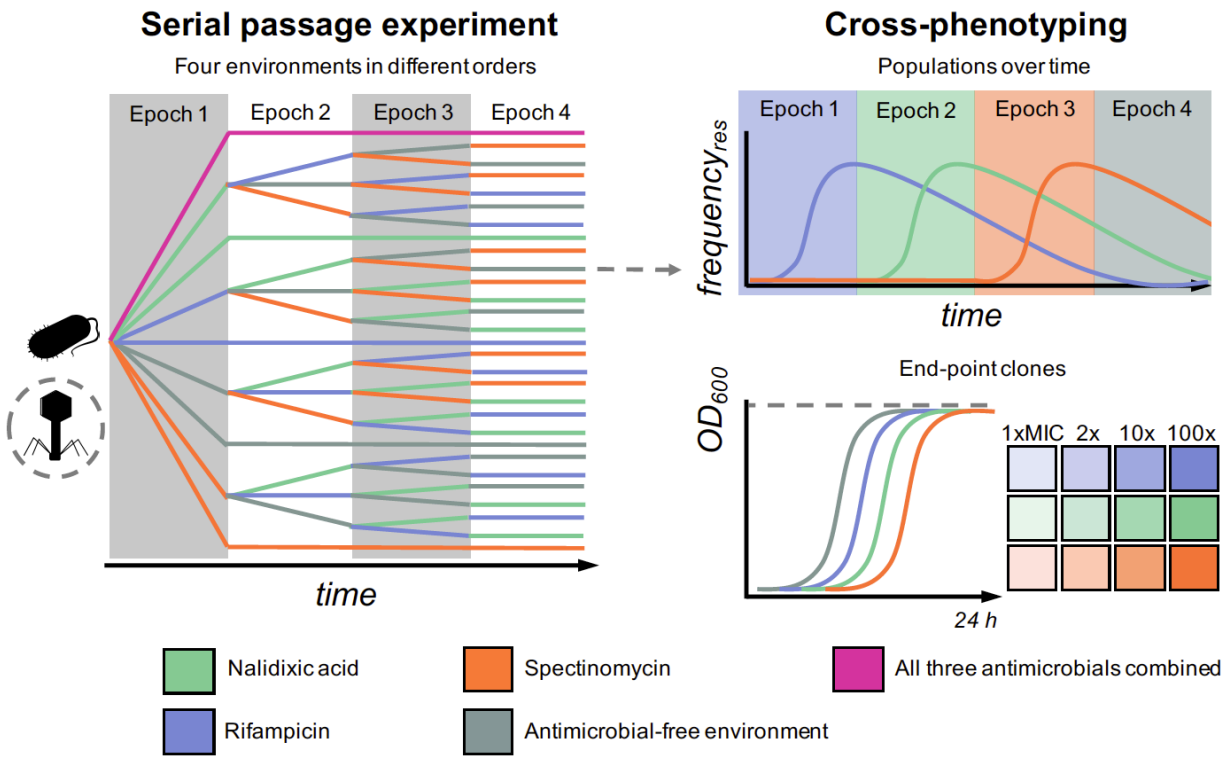

Fig. 1. Overview of experimental design. The main experiment on the left was a 48-day serial passage experiment where initially isogenic *E. coli* was subjected to a control environment without antimicrobials; monotherapy or combination therapy with three different antimicrobial compounds; and alternating therapy regimes encompassing all permutations of four environments, including the three antimicrobials and one antimicrobial-free environment. The full experiment was repeated with and without initial introduction of phage representing an alternative type of antimicrobial agent. Each unique treatment combination was replicated 16 times, amounting to a total of 928 independent populations, which were cross-phenotyped over time against resistance to each of the antimicrobials. In addition, a single dominant clone was isolated from all surviving end-point populations ($N = 900$) and phenotyped for growth (optical density, OD, at 600 nm after 24 h culture) at several concentrations of each antimicrobial. To investigate the underlying molecular evolution, a subset of 235 clones representing different therapy regimes and divergent resistance outcomes were also subjected to whole-genome sequencing.

Resistance dynamics, end-point outcomes and recurrent mutations differ depending on antimicrobial regime

The three antimicrobial compounds used target different key cellular structures in bacteria: DNA gyrase (*nalidixic acid*, a quinolone-like naphtyridone antibiotic), RNA polymerase (*rifampicin*, a rifamycin antibiotic) and the small subunit of the ribosome (*spectinomycin*, an aminoglycoside-like aminocyclitol antibiotic). All four antimicrobial agents, including the three chemical compounds at subinhibitory levels (0.5 × MIC) and phage (T4), differed considerably in bacterial resistance evolutionary dynamics depending on the therapy regime.

Nalidixic acid resistance: Nalidixic acid (quinolone class) alone or in combination with the two other compounds caused resistance to nalidixic acid to evolve in a large proportion of the replicate populations over the course of 48 days (Fig. 2A; antimicrobial regime, $P < 0.001$; for full results, see Table S1). However, selection was not strong enough to cause a marked increase in the number of resistant populations under monotherapy within a 12-day window, representing the

length of a single exposure epoch in the alternating therapy protocol. Nevertheless, selection for resistance did occur within a 12-day window in combination therapy. Moreover, the proportion of replicate populations displaying resistance was a poor proxy for the allele frequency of resistance within the populations, as single dominant clones isolated from the populations at the experimental end-point (likely to represent the dominant genotype) displayed less resistance than the population as a whole (Fig. 2C; Table S2). This indicates that most of the populations displaying resistance had it as part of a minor clone.

Genomic data from the clones supported these findings such that nalidixic acid therapy was associated with a small number of nonsynonymous mutations overall (median of 0 mutations in monotherapy in the absence of phage, equivalent to control environment) as well as a relatively small number of recurrent mutational targets occurring only in a small proportion of the isolates (Fig. 2G). This is consistent with major clones lacking resistance in nalidixic acid monotherapy in the absence of phage (Fig. 2C). These targets include *acrR* (encoding HTH-type transcriptional regulator), *rfaQ* (lipopolysaccharide core heptosyltransferase) and ECB_RS03400 (putative phosphoglucomutase). Among these genes, *acrR* has been previously implicated in quinolone resistance (*18*), while the product function (LPS biosynthesis) and previous findings suggest that *rfaQ* (*19*) and phosphoglucomutase (*20, 21*) may be associated with resistance to both quinolone and phage. Together these findings show that nalidixic acid causes strong selection for resistance in mono- and combination therapy while causing weak selection for resistance in alternating therapy.

Rifampicin resistance: Rifampicin (rifamycin class) alone or in combination with the other two compounds imposed strong directional selection for resistance at the population level, and already within a 12-day epoch window (Fig. 2B; antimicrobial regime, $P < 0.001$; for full results, see Table S3). Data on resistant populations was a relatively good proxy for allele frequency of resistance within populations, as dominant clones isolated from resistant end-point populations were also likely to exhibit a resistant phenotype (Fig. 2D; Table S4). This indicates that most of the populations displaying resistance had it as part of a major clone. However, the clone data showed less resistance for alternating therapy compared to the population data (Fig. 2B end-point vs. Fig. 2D). This indicates that resistance had lower allele frequencies in alternating therapy compared to mono- and combination therapy. The vast majority of clones from the monotherapy environment had nonsynonymous mutations (median of 1.5 mutations in the absence of phage) in the gene *rpoB* (β subunit of RNA polymerase) known to produce rifampicin resistance (*22*), and the presence of mutations in this gene was almost exclusively associated with a resistance phenotype (Fig. 2G). In addition, five other genes (*galU*, *infB*, *marR*, *mreC* and *mrdB*) were recurrently mutated only in the presence of rifampicin (for details on association with resistance, see Supplementary text). Together these findings show that rifampicin causes strong selection for resistance, with selection strength weakening in the following order: mono-, combination and alternating therapy.

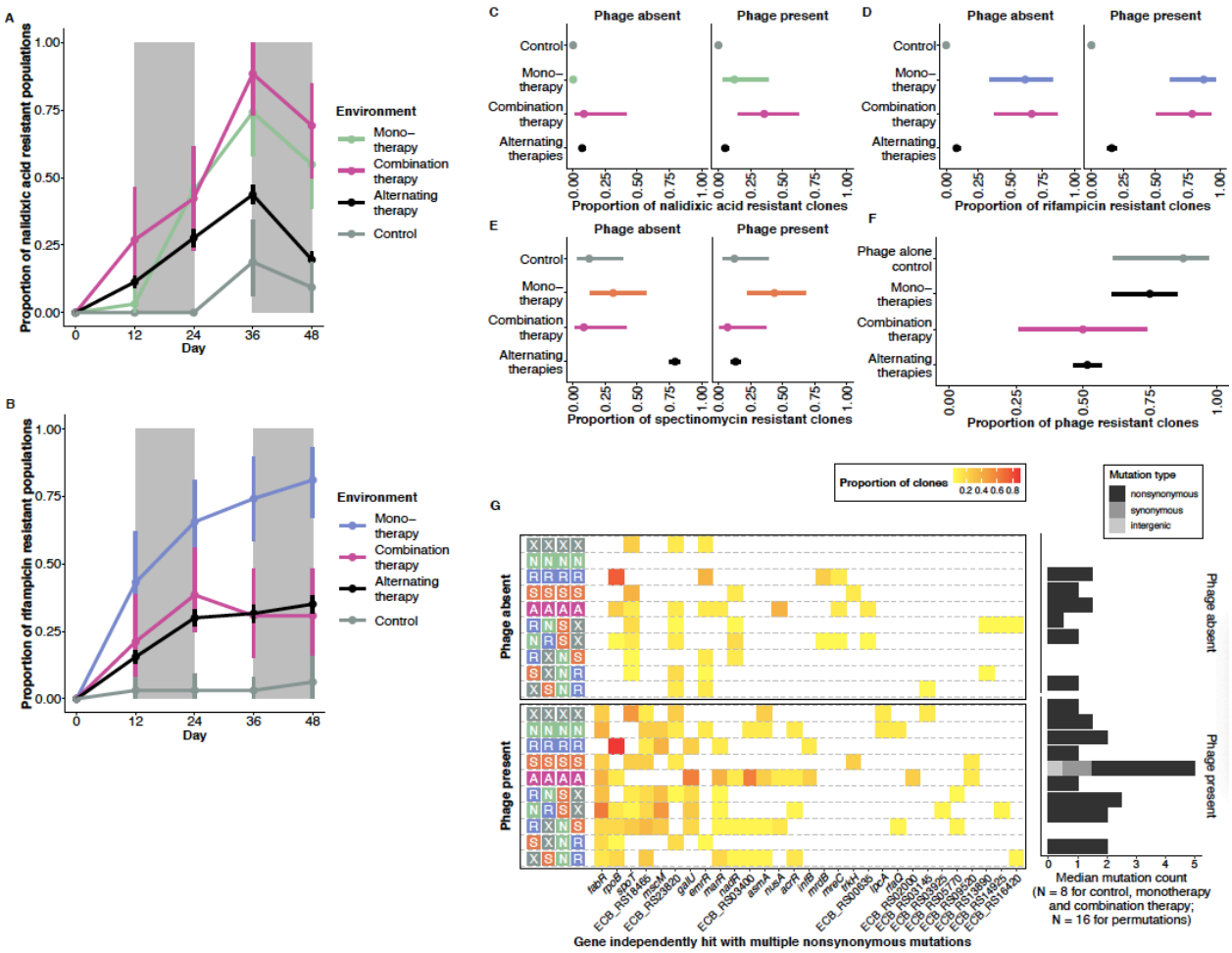

Fig. 2. Contingency of phenotypic outcome and mutation landscape on antimicrobial regime. (A) and (B) show resistance dynamics over time for nalidixic acid and rifampicin, respectively (mean ± bootstrapped 95 % confidence intervals; 32 replicates per mean data point; data in presence and absence of phage pooled). Grey rectangles denote epoch boundaries for alternating therapy protocols. (C), (D), (E) and (F) show resistance outcomes to nalidixic acid, rifampicin, spectinomycin and phage T4, respectively, for clones isolated from each population at the experimental end-point (logistic regression expected value ± 95 % confidence intervals; 16 replicates per mean data point). Resistance has been quantified as a binary variable and indicates the ability to grow at levels exceeding the minimum inhibitory concentration of the ancestral bacterial strain. (G) Mutational landscape. The heat map on the left shows the proportion of sequenced clones containing a nonsynonymous (or infrequently synonymous) mutation in a gene recurrently hit in the dataset. The genes have been ordered by total number of hits. The bar plot on the right shows the median mutation count for clones in each history (lack of bar indicates median of 0 mutations). The *y*-axis labels indicate the antimicrobial therapy protocol with the following encoding for each of the four 12-day experimental epochs: X = antimicrobial-free environment; N = nalidixic acid; R = rifampicin; S = spectinomycin; A = all three antimicrobial compounds combined.

Spectinomycin resistance: Spectinomycin (aminoglycoside class) monotherapy, either in the presence or absence of phage, led to higher mean spectinomycin resistance prevalence among end-point clones compared to the antimicrobial-free control and combination therapy environments, although resistance increased markedly only in the alternating therapy environment in the absence of phage (Fig. 2E; antimicrobial regime, $P < 0.001$; for full results, see Table S5). We were unable to obtain robust population-level time series data for spectinomycin, as the data was flooded in

(majority of populations gave) positive signal masking any potential treatment effect. There are a number of factors that could enable population growth in our selective conditions, resulting in a positive resistance signal despite all or most of the cells remaining sensitive. Resistance may occur only in a subset of the population owing to a low selection coefficient (*9*), reversible amplifications (*23, 24*), or plastic gene regulatory changes induced by antimicrobial stress (e.g. SOS or stringent response) (*25, 26*). Three genes, *nadR* (transcriptional regulator), *trkH* (potassium uptake protein) and ECB_RS09520 (putative carboxyl-terminal processing protease), were recurrently mutated specifically in the presence of spectinomycin (median of 1 nonsynonymous mutation in monotherapy), with only *trkH* reaching high (close to 0.5) frequencies (Fig. 2G). Among these, *trkH* has been previously implicated in aminoglycoside resistance (*27, 28*), while *nadR* (*29*) and proteases (*30*) can, among other functions, be related to the bacterial stress response. Together these findings suggest that spectinomycin monotherapy may cause weak selection pressure for resistance. As much higher resistance levels occur in a subset of the alternating therapy environments, with a shorter spectinomycin selective window, these are expected to arise from the other agents (see following results section).

Phage resistance: The majority of bacteria exposed to the phage had a resistant phenotype still at the experimental end-point (i.e. 48 days after phage introduction) (Fig. 2F). Antimicrobial regime significantly affected phage resistance levels ($p < 0.001$; for full results, see Table S6), which showed a decreasing trend from the control environment (approx. 88 % resistance) through monotherapy (approx. 75 % resistance) to combination or alternating therapy (approx. 50 % resistance). This indicates that combination and alternating therapies constrained either selection for or maintenance of phage resistance. Phage exposure caused recurrent mutations in several genomic targets almost exclusively associated with phage resistant phenotypes, with most targets exhibiting low to moderate (<0.5) prevalence among isolates (Fig. 2G). This indicates that resistance to the phage T4 has a wide target in *E. coli* B. This is consistent with earlier studies showing that membrane modifications preventing phage adsorption represent a highly common resistance mechanism of bacteria against virulent phages and can typically be achieved by mutations in a number of genes affecting membrane structure and components (*31*). Moreover, the phage caused an increase of one nonsynonymous mutation in the median mutation count of the clones, suggesting that individual mutations rather than several mutations in combination were required for phage resistance. The potential phage resistance targets discovered in this study encompass 14 genes: *acrR*, *asmA*, *fabR*, *galU*, *infB*, *lpcA*, *mscM*, *rfaQ*, ECB_RS0200, ECB_RS03400, ECB_RS03925, ECB_RS05770, ECB_RS09520, and ECB_RS18465 (Fig. 2G; see Supplementary text for associations between these genes and resistance phenotypes). Together these findings show the phage causes strong selection for resistance, with selection strength weakening (similar to rifampicin) in the following order: mono-, combination and alternating therapy. Overall, different therapies produced different evolutionary effects on the bacteria, both in terms of genetics and the extent of resistance observed in the populations.

Pleiotropic and fitness effects of strong selective agents, rifampicin and phage, modulate overall resistance outcome

Unexpectedly, rifampicin monotherapy resulted in an increased probability of nalidixic acid and spectinomycin resistance, specifically in the absence of phage (Fig. 3A). When comparing control and rifampicin monotherapy environments with and without phage, nalidixic acid resistant clones only occurred in rifampicin monotherapy environments without phage. Spectinomycin resistance,

in turn, occurred in a small subset of the populations also in the control environments but was extremely prevalent in the rifampicin monotherapy environment without phage (rifampicin monotherapy vs. control, $P = 0.039$; phage presence, $P < 0.001$; interaction, $P = 0.002$; for full results, see Table S7). Moreover, spectinomycin resistance levels were much higher in alternating therapy protocols in the absence of phage compared to spectinomycin monotherapy. We hypothesized that both observations could result from pleiotropic effects of mutations selected by rifampicin, with either mutational targets being altered or the pleiotropic effect being modulated by the presence of phage. In line with the latter explanation, we found that 6/8 clones containing *rpoB* mutations (producing rifampicin resistance) and unexposed to the phage displayed resistance to spectinomycin. Conversely, only 4/17 clones with *rpoB* mutations in the presence of phage displayed resistance to spectinomycin. We also found a similar pattern of antimicrobial cross-resistance depending on phage resistance for two other genes: the stringent response gene *spoT* which was mutated across experimental treatments and the previously identified spectinomycin-selected gene *nadR* (Fig. 3B). Consequently, clones containing mutations in either *nadR*, *rpoB* or *spoT* had a high likelihood of exhibiting a spectinomycin resistance phenotype conditioned on occurring in a phage susceptible genomic background (ANOVA for binomial generalized linear model: presence of mutations in *nadR*, *rpoB* or *spoT*, $\chi^2_{1,233} = 1.90$, $P = 0.17$; phage resistance, $\chi^2_{1,232} = 25.7$, $P < 0.001$; presence of mutations in *nadR*, *rpoB* or *spoT* × phage resistance, $\chi^2_{1,231} = 7.01$, $P = 0.008$).

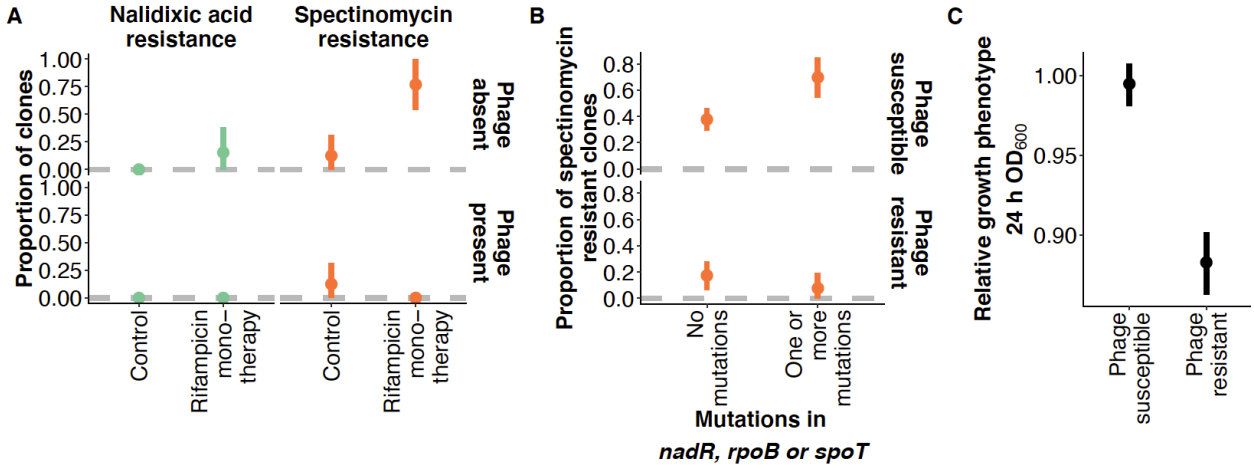

Fig. 3. Pleiotropy and fitness effect of resistance. (A) Nalidixic acid and spectinomycin resistance after 48 days of rifampicin monotherapy (mean ± bootstrapped 95 % confidence intervals). (B) Influence of phage resistance on whether mutations in the genes *nadR*, *rpoB* or *spoT* produce a spectinomycin resistant phenotype (mean ± bootstrapped 95 % confidence intervals). The data is for clones from phage-exposed environments (for which phage resistance phenotype was determined). (C) Fitness effect of phage resistance (mean ± bootstrapped 95 % confidence intervals). Fitness has been quantified as optical density (OD) at 600 nm wavelength after 24 h culture in liquid medium. The value has here been related to the mean growth of the clones from the control treatment (absence of antimicrobials and phage). The data for all the figures is for clones isolated from populations at the experimental end point (N_{total} = 900), with subset treatments or phenotypes included in a particular analysis indicated in the figure or legend.

Furthermore, we found that all *mreC* mutants (selected by rifampicin) as well as a single *mreD* mutant were multidrug resistant. Two of three *mreC* mutants (containing the same frameshift mutation, Glu291fs) and the *mreD* mutant were resistant to all three drugs, while a single *mreC* mutant

(Val46Gly) was resistant to both nalidixic acid and spectinomycin but remained susceptible to rifampicin. Mutations in *mreC* and *mreD*, whose products work in concert to determine cell shape and elongation, did not occur in the presence of phage. In turn, mutations in *marR* selected by rifampicin that occurred only in the presence of phage also resulted in nalidixic acid resistance. These mutations could therefore explain the increased probability of nalidixic acid resistance in the presence of rifampicin. In addition, we found that phage resistance, associated in particular with mutations in *fabR*, *galU* and ECB_RS18465, had a strong fitness cost as indicated by reduced bacterial growth after 24 culture (88.8 % growth of phage sensitive clones) in the absence of the phage or antimicrobial compounds (ANOVA for linear model: phage susceptibility, $F_{1,898} = 84.1$, $P < 0.001$) (Fig. 3C). Together these observations suggest that nalidixic acid and spectinomycin resistance dynamics were to a large extent driven by the other two selective agents (rifampicin and phage) imposing stronger selection through the following three mechanisms: i. cross-resistance (*i.e.* pleiotropic) mutations selected in the presence of rifampicin; ii. the effect of phage on the strength of selection for antimicrobial resistance and the targets of antimicrobial resistance mutations; iii. the loss of the antimicrobial resistance phenotype of particular mutations in a phage resistant background. Cases ii. and iii. may be related to the strong fitness-impairing consequence of phage resistance.

Modifying effects of strong selective agents largely explain differences between alternating therapy protocols

The resistance evolutionary patterns described above largely determined differences in resistance levels between the alternating therapy protocols. First, rifampicin which was the strongest selective antimicrobial compound caused resistance to occur as a function of exposure epoch mainly by selecting for *rpoB* mutations (Figs 4C & S1B; rifampicin exposure epoch, $p = 0.008$; for full results, see Table S8). Second, nalidixic acid resistance occurred at a much lower level in general (Figs 4A,B & S1A). It occurred as a function of rifampicin exposure in the absence of phage where selection by nalidixic acid was weak and rifampicin selected for low levels of nalidixic acid cross-resistance by *mreC* mutations (Figs 4A & S1A; nalidixic acid exposure epoch, $P = 0.48$; rifampicin exposure epoch, $P = 0.003$; for full results, see Table S9). In the presence of phage, however, nalidixic acid resistance was more strongly driven by nalidixic acid exposure selecting for *acrR* and ECB_RS03400 mutations (Figs 2G, 4B & S1B; nalidixic acid exposure epoch, $P = 0.007$; rifampicin exposure epoch, $P = 0.22$; for full results, see Table S10). This is consistent with the bacterial cells experiencing stronger selection for nalidixic acid resistance in the presence of both phage and nalidixic acid compared to nalidixic acid alone. Finally, in line with cross-selection by rifampicin, spectinomycin resistance level was influenced by both the spectinomycin and rifampicin exposure epochs, although being mainly determined by the presence of phage (Figs 3B & S1C; spectinomycin exposure epoch, $P = 0.012$; rifampicin exposure epoch, $P < 0.001$; phage presence, $P < 0.001$; for full results, see Table S11).

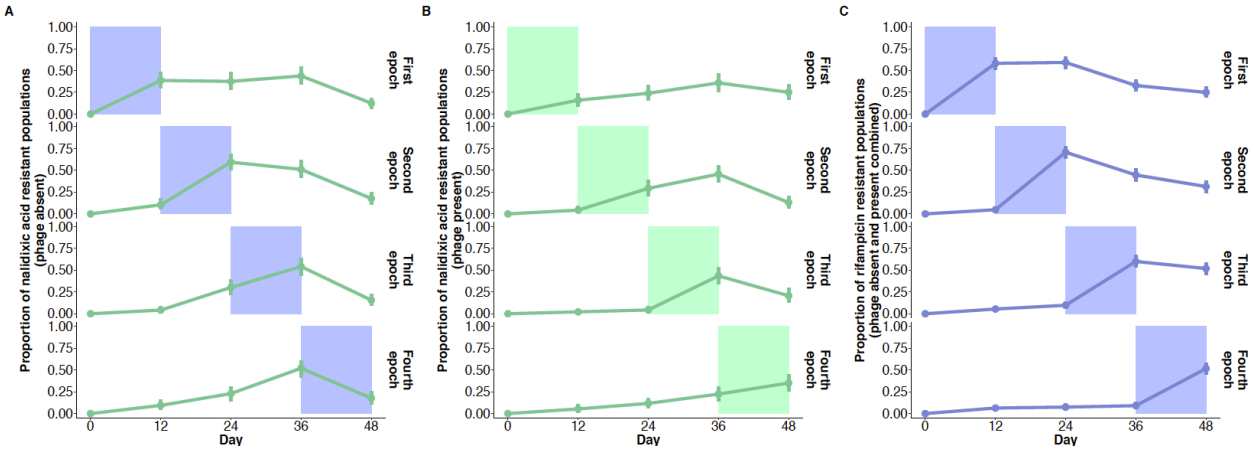

Fig. 4. Antimicrobials driving resistance evolution in alternating therapy regimes. (A) Nalidixic acid resistance over time in the absence of phage as a function of rifampicin exposure epoch. (B) Nalidixic acid resistance over time in the presence of phage as a function of nalidixic acid exposure epoch. (C) Rifampicin resistance over time as a function of rifampicin exposure epoch (both in presence and absence of phage which had no effect on selective antimicrobial as it did for nalidixic acid). All the data is shown as mean resistance ± bootstrapped 95 % confidence intervals, and is based on $N = 928$ populations. The shaded area indicates the relevant (antimicrobial color code) exposure epoch.

Because high levels of spectinomycin resistance in the absence of phage accounted for a large proportion of resistance data, multidrug resistance (here referring to resistance to antimicrobial compounds and excluding resistance to the phage) was more likely to occur in the absence of phage despite the phage exacerbating selection for rifampicin and nalidixic acid resistance. As nalidixic acid selection was weak, most cases of multidrug resistance were cases of rifampicin-spectinomycin cross-resistance. Notably, three out of five among the sequenced clones displaying resistance to all three agents contained mutations in the cell-shape determining genes *mreC* and *mreD*. Although rifampicin resistance levels began to decay after the rifampicin exposure epoch, likely owing to a fitness cost of rifampicin resistance, the effect was too weak to introduce a clear history dependence effect on multidrug resistance levels. Therefore, differences in the resistance outcome between the alternating therapy protocols were mostly accounted for by the following factors: differences in selection strength between the agents; pleiotropic effects of rifampicin resistance; and modifying effects of phage exposure on antimicrobial resistance phenotypes and evolution.

Inference of ecological past and predictability of evolutionary future are strongest for driver agents

The strong selective agents (rifampicin and phage) largely accounting for the multidrug resistance landscape, correspondingly, exhibit the strongest predictive power regarding the past drug exposure and future resistance outcome of the bacterial populations (Fig. 5A). As expected based on the strong fitness consequence of phage resistance, machine learning (random forest) models were able to predict past phage exposure with high accuracy based on growth data from end-point clones in the absence or presence of different levels of the experimental antimicrobials. The same data could also be used to train a model to precisely predict the rifampicin exposure epoch, consistent

with rifampicin resistance levels both decaying after exposure and influencing the overall resistance phenotypes. Conversely, in line with expectations, high-accuracy predictive models could not be constructed for the exposure epoch of the weak selective agent nalidixic acid or cross-selected agent spectinomycin. These factors were also seen as in modest predictive power of models predicting the full antimicrobial exposure sequence (*i.e.* ecological past). Therefore, the ability to predict the ecological past from the current phenotypic state is increased by high selection coefficients and strong fitness effects of resistances and can be obscured by low selection coefficients and pleiotropy.

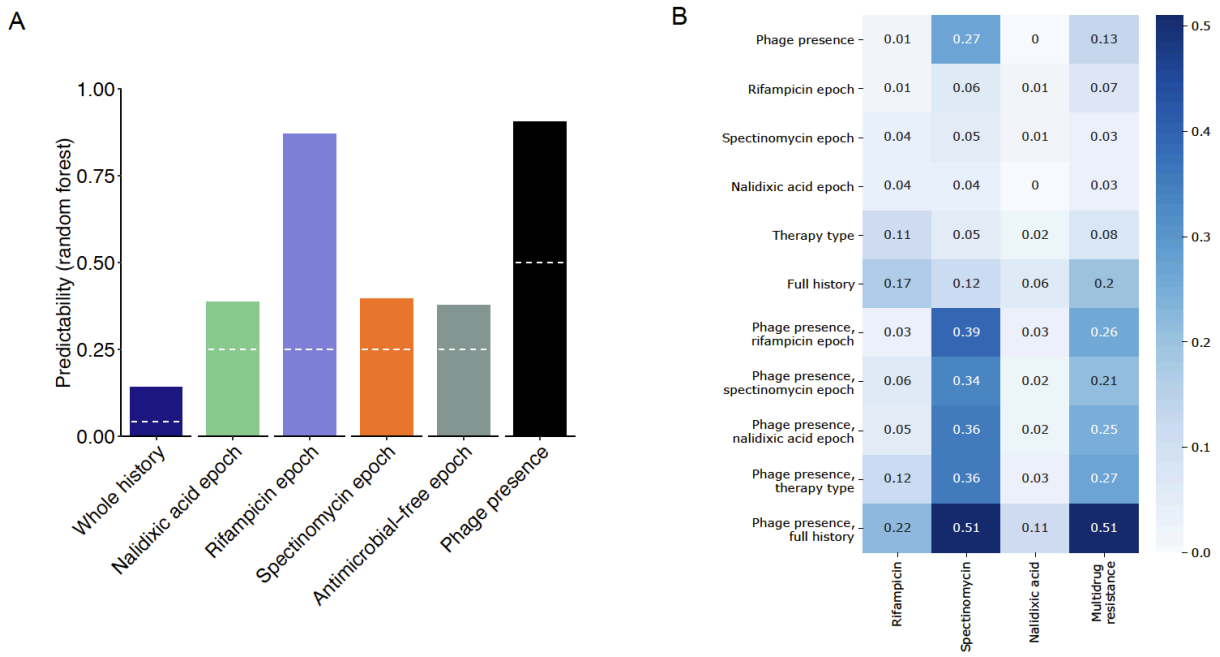

Fig. 5. Predictability of past drug exposure and future resistance evolution in alternating drug therapy regime. (A) Degree of predictive power obtained for antimicrobial and phage exposure past based on phenotypic data from populations and clones at the end-point of 48-day serial passage experiment. The white dashed line indicates the level of predictability of the estimated factor by chance. (B) Mutual information between exposure history (rows) and end-point clone antimicrobial resistance phenotype states (columns). The exposure histories are described at various levels of detail ranging from coarse (phage status and epoch of exposure to an antimicrobial separately) to full information (phage status and antimicrobial epoch order considered simultaneously).

We further quantified information theoretically the relationship between exposure histories and each of the end point phenotypes individually by evaluating their mutual information (Fig. 5B). A large value of mutual information between an environment and a phenotype indicates that knowing one substantially removes uncertainty about the other. Phage and spectinomycin exhibited strong mutual information (0.27 std 0.03) and adding the detail of the antimicrobial exposure order further increased it to 0.51 std 0.03. For nalidixic acid and rifampicin, knowing whether the exposure history had phage or not carried little information. However, for both compounds, increasing the detail of the antimicrobial exposure order carried information. In contrast to the random forest

modelling results, we noticed that the epoch of exposure to rifampicin did not greatly reduce uncertainty for the individual end-point resistance states. This is due to the random forest model exploiting both population and clone data at multiple MIC values – beyond binary (quantitative OD value instead of 0 for susceptible and 1 for resistant) – as well as using the joint phenotype, respect to all compounds, as its basis for predicting the past exposure environment.

Discussion

Inspecting differences in antimicrobial resistance dynamics between mono-, combination and alternating antimicrobial protocols, we found that combination therapy led to a high prevalence of resistances and associated mutations. These patterns were likely influenced by our use of subinhibitory antimicrobial selection. At high (super-MIC) antimicrobial levels, we would expect both ecological conditions (high population extinction probability) and genomic factors (requirement for presence of multiple resistances in standing genetic variation for evolutionary rescue) to impose stronger constraints on resistance evolution (*13*). Nevertheless, the pleiotropic effects of mutations occurring in the presence of rifampicin which caused strong directional selection for its respective resistance rendered rifampicin monotherapy a similarly potent enhancer of multidrug resistance. Moreover, the presence of phage, causing strong selection for phage resistance associated with a major impairment in bacterial growth, was a powerful modulator of antimicrobial resistance: on the one hand, removing the MIC-increasing effect of mutations causing resistance to one antimicrobial (spectinomycin) and, on the other, increasing directional selection for resistance by two antimicrobials (nalidixic acid and rifampicin). The prevalence of pleiotropic effects of antimicrobial resistance mutations (*4*) and synergistic or antagonistic effects of phage-antimicrobial combinations on resistance trajectories (*12, 32*) suggest that our results may reflect a more widespread phenomenon: Particular, strong selective agents can be equally or more critical for multidrug resistance evolution compared to the individual agents and their direct resistance selective properties within a therapy regime.

The strong selective agents (rifampicin and phage) also modulated resistance dynamics in our alternating therapy protocols, with both the rifampicin exposure epoch and the presence of phage exposure influencing the prevalence of resistance at the experimental end-point to all three antimicrobials used. The resistance outcome for the two other antimicrobials was also a function of their own exposure epoch and was either associated with weak selection (nalidixic acid) or co-selection by a variety of experimental conditions (spectinomycin). Therefore, the predictability of the ecological past based on end-point phenotypes or the future evolutionary state based on drug exposure sequence was lower for them compared to the two driver agents in our system. This indicates that pleiotropic effects of adaptive mutations can obscure the ability to predict resistance outcomes in sequential drug therapies and thus complicate therapy optimization. Notably, most cases of resistance in our study were low-level resistance (ability to grow at the MIC of the ancestral strain) owing to subinhibitory selection. The independent selective effect of constituent drugs may be stronger, and predictability for alternating therapies therefore higher, at high-level clinical antimicrobial concentrations (*7*). Alternatively, fitness trade-offs and epistasis are likely to be stronger for high-level resistance mutations reaching fixation during each antimicrobial exposure period, which may canalize resistance pathways and increase divergence in resistance outcomes between alternating protocols. If this occurs by highly protocol-specific mutational pathways, this may decrease the capacity of general factors to predict the resistance outcome compared to our setup. Despite the variability in resistance outcomes between protocols, we nevertheless found

overall decreased directional selection for and thereby prevalence of resistance in alternating compared to mono- and combination therapy. This supports the general utility of alternating protocols in decreasing resistance levels and extending the shelf-life of existing antimicrobials (*16, 17*).

The driver agents modified the interplay between selection and the epoch length by exacerbating selection and thus partially removing the desired filtering effect of epochs to resistance evolution. As the driver agents affected resistance evolution to both directions, assessing their overall impact for a specific therapy requires experimentation. Clearly, identifying and testing the impact of such driver agents has potential for therapy optimization and their efficient usage should be studied further using eco-evolutionary control theory (*33*). Intriguingly, driver agents do not necessarily need to be antimicrobials, for instance, the effects of stress environments can be further modulated by inhibiting global regulators (*34*).

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Data and materials availability: Sequence data will be deposited in NCBI SRA. All code and pre-processed data needed to reproduce the downstream analyses and figures will be available vie Dryad/GitHub.