# Alternating antibiotic treatments constrain evolutionary paths to multidrug resistance

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**Alternating antibiotic therapy, in which pairs of drugs are cycled during treatment, has been suggested as a means to inhibit the evolution of de novo resistance while avoiding the toxicity associated with more traditional combination therapy. However, it remains unclear under which conditions and by what means such alternating treatments impede the evolution of resistance. Here, we tracked multistep evolution of resistance in replicate populations of *Staphylococcus aureus* during 22 d of continuously increasing single-, mixed-, and alternating-drug treatment. In all three tested drug pairs, the alternating treatment reduced the overall rate of resistance by slowing the acquisition of resistance to one of the two component drugs, sometimes as effectively as mixed treatment. This slower rate of evolution is reflected in the genome-wide mutational profiles; under alternating treatments, bacteria acquire mutations in different genes than under corresponding single-drug treatments. To test whether this observed constraint on adaptive paths reflects trade-offs in which resistance to one drug is accompanied by sensitivity to a second drug, we profiled many single-step mutants for cross-resistance. Indeed, the average cross-resistance of single-step mutants can help predict whether or not evolution was slower in alternating drugs. Together, these results show that despite the complex evolutionary landscape of multidrug resistance, alternating-drug therapy can slow evolution by constraining the mutational paths toward resistance.**

experimental evolution | antibiotic resistance | multidrug therapy | drug cycling | collateral sensitivity

The prevalence of antibiotic resistance continues to rise (1–3), with over 2 million antibiotic-resistant infections per year in the United States alone (4). Resistant mutants can arise de novo within the course of a single long-term infection (5, 6), particularly under low drug doses resulting from poor drug compliance or incomplete penetration of drug to all tissues (7). Even highly multidrug-resistant bacteria can gain additional resistance de novo (8), threatening the efficacy of last-line drugs. In the face of such multidrug-resistant bacteria and a slowing pace of drug development (2, 9), treatment regimens that minimize the risk of resistance are needed as a complement to antibiotic stewardship (9).

Drug mixtures have been used to slow the emergence of resistance with some success (10). It is expected that rate of evolution in mixed treatment is typically slower than in single drugs, because mutations conferring resistance to only one of the individual drugs may not provide a large advantage in the multidrug environment (11). The possibility and extent of reduction in the rate of evolution in mixed-drug treatments therefore depend on the interaction between the drugs and on the level of positive or negative cross-resistance among them (12–18). However, combination therapy is limited by prohibitive toxic side effects (19–21). Many drugs, especially the last-resort drugs used for multidrug-resistant infections, like colistin, are toxic when used for long periods of time (8, 20). Increased total drug dosage and the addition of other drugs can exacerbate this toxicity, especially among the hospitalized patients most in need of combination therapy.

One underexplored strategy to slow the evolution of antibiotic resistance is alternating therapy, in which drugs are administered one at a time with periodic switching. Although the strategy of alternating (or cycling) drugs in entire hospital wards has long been debated (16, 22–26), rapidly alternating drug regimens in individual patients with long-term bacterial infections have received little attention. A recent study showed that *Escherichia coli* cells with a mutation conferring resistance to one drug and collateral sensitivity to a second drug (negative cross-resistance) are outcompeted by wild-type cells in that second drug, suggesting that cycling such drugs may slow the acquisition of resistance (16). In addition, alternating drug regimens may avoid the toxicity of traditional combination therapy. However, it remains unclear whether alternating therapy is effective long-term and how such treatments affect adaptive mutational paths to resistance.

Here, we characterized the rate of evolution of resistance in *Staphylococcus aureus* under single-, mixed-, and alternating-drug treatments for three drugs representing distinct antibiotic classes. We then sequenced the evolved populations to gain a genotypic understanding of adaptation in single- and multiple-drug environments. Finally, we determined the cross-resistance profiles of a separate set of single-step mutants exposed to each of the three drugs, exploring whether the effectiveness of alternating drugs stems from diminished selection for mutations conferring resistance to one drug but increased sensitivity to the other.

## Results

**Alternating Drugs Impedes Evolution of Resistance.** A total of 120 independent populations of *S. aureus* were subjected to single-, mixed-, and alternating-drug treatments comprised by subsets of three drugs (Fig. 1). We serially passaged bacterial populations

### Significance

**Antibiotic resistance is a growing threat, but the pace of drug discovery remains slow. Combination therapy can inhibit the emergence of de novo resistance but is often too toxic for long-term use. Alternating treatments, in which drugs are used sequentially with periodic switching, have been proposed as a substitute, but it remains uncertain when and how they slow the evolution of resistance. Using experimental evolution and whole-genome sequencing, we find that alternating drugs slows the rate of increase in resistance compared with single-drug treatments, by constraining resistance mutations with trade-offs in resistance to a second drug. Thus, drug combinations can exploit these trade-offs to slow the evolution of resistance, even when the drugs are not used simultaneously.**

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Data deposition: The sequences reported in this paper have been deposited in the National Center for Biotechnology Information Sequence Read Archive database, www.ncbi.nlm.nih.gov/sra (accession no. SRP045373, BioProject no. PRJNA257510).

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in twofold gradients of antibiotic concentration daily for 22 d (Fig. 1*A*), dynamically selecting for high levels of resistance (27). At the end of each growth cycle (20 h), the culture at the highest drug concentration permitting bacterial growth ($OD_{600} > 0.2$) was propagated into a fresh antibiotic gradient (1:100 dilution). To account for the inherent stochasticity of evolutionary adaptation and enable comparison between regimes, we followed 10 replicate populations for each drug treatment.

We monitored changes in resistance using two methods. First, each population's resistance at each time point was inferred from the drug concentration in the well chosen for propagation (Fig. S1). Additionally, to facilitate direct comparison across treatments, we measured the single-drug resistance levels of all evolved populations (*Materials and Methods*). These retrospective measurements were consistent with the inferred resistance levels (Fig. S2).

We used three drugs with different mechanisms of action and resistance (Fig. 1*B*): trimethoprim (TMP), a dihydrofolate reductase inhibitor (28); neomycin (NEO), an aminoglycoside targeting the ribosome (29); and ciprofloxacin (CIP), a fluoroquinolone targeting the DNA gyrase/topoisomerase complex (30). Aminoglycosides and fluoroquinolones have been used, either singly or in combination with other drugs, to treat *S. aureus* infections (31, 32), whereas TMP has been considered for use in combination with sulfamethoxazole as an alternative to vancomycin in treating methicillin-resistant *S. aureus* (33).

In addition to these three single-drug treatments, we tested mixed- and alternating-drug treatments for each pair of drugs (Fig. 1*C*). For mixed-drug treatments, we used a gradient of a mixture of the two drugs, at a fixed-ratio of approximately equal drug inhibition. For alternating-drug treatments, we switched between the two single-drug gradients daily, starting with each drug. Although inferred resistance trajectories appeared to differ depending on the starting drug (Dataset S1), we found no significant effect of the starting drug on final resistance levels (Fig. S3) ($P > 0.3$, all $t$ tests), and have therefore combined the two drug orders for subsequent analyses.

Every alternating-drug treatment slowed the overall rate of evolution compared with treatment with one of the corresponding single drugs alone (Fig. 2). Because alternating-drug populations were exposed to each drug for only 11 of the 22 d, we compared their final resistances to those of single-drug populations at day 11. Alternating TMP and NEO slowed the rate of acquisition of TMP resistance (by 41%, $P = 0.0003$) (see *Materials and Methods* for a description of statistical analyses); alternating NEO and CIP slowed CIP resistance (by 54%, $P = 0.0003$); and alternating TMP and CIP slowed TMP resistance (by 40%, $P = 0.0033$). In some cases, the slowing effect was steady for the duration of the evolution, whereas in others the resistance was slowed primarily in the first 2 d.

Compared with the corresponding mixed treatment, the alternating treatment was equally or less effective. As expected, the mixed treatments significantly slowed the evolution of resistance compared with any one of the two drugs used for the same length of time (Fig. S4), and even compared with an expectation for the resistance after two sequential 11-d single-drug treatments (Fig. 2). Alternating TMP and CIP was indistinguishable from the mixed treatment, and alternating NEO and CIP resulted in less CIP resistance but more NEO resistance than the mixture (Fig. 2). However, alternating drugs was unambiguously less effective in the TMP-NEO drug pair. Thus, alternating between two drugs impedes evolution of resistance, sometimes as effectively as a simultaneous mixture of the same two drugs.

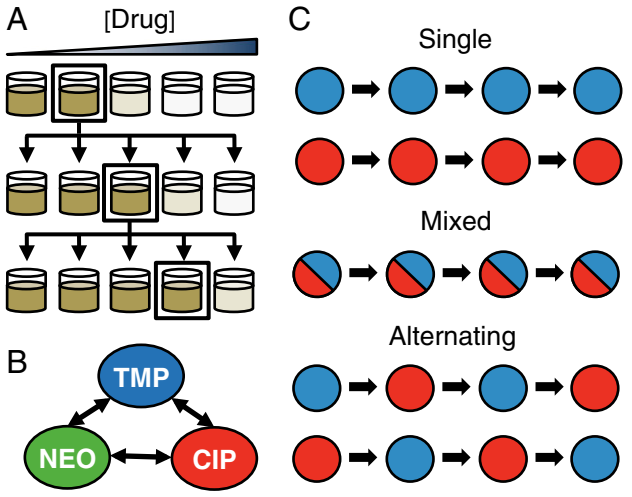


**Fig. 1.** Experimental evolution of antibiotic resistance under multidrug treatments. (*A*) Each population of *S. aureus* was inoculated into a series of wells with a gradient of drug concentrations. After 20 h, the well with the highest drug concentration permitting bacterial growth ($OD_{600} > 0.2$) was used to inoculate the next cultures. This procedure was repeated for 22 d in 10 replicate populations per drug treatment. (*B*) Three antibiotics and their pairwise combinations were studied: TMP (blue), NEO (green), and CIP (red). (*C*) For each drug pair, we tested each single drug individually, a mixed treatment using a fixed ratio of the two drugs, and alternating treatments with daily switching between the two single drugs, starting with either drug.

**Alternating Drugs Constrains Mutational Paths to Resistance.** To gain a genotypic view of evolution in alternating drugs, we sequenced the genomes of the evolved populations (*Materials and Methods*). We identified a total of 515 mutations across all 120 populations (Dataset S2). These mutations showed a strong signal for positive selection (dN/dS = 6.2, 95% confidence interval = 3.4–12.5) (*SI Materials and Methods*) and a high degree of parallelism: 53% of mutations were found in genes that were mutated in at least four populations (Fig. S5).

These frequently mutated genes included known and novel antibiotic-resistance genes. As expected, the "primary" resistance genes—the genes most commonly mutated among single-drug populations—were known drug-target genes (Fig. 3*A*): *folA* (dihydrofolate reductase) for TMP (28), *fusA* (elongation factor G) for NEO (34), and *grlA* (DNA topoisomerase IV) for CIP (35). In addition, we found many mutations in genes not previously associated with resistance, particularly in the multidrug treatments: there were 25 mutations in *rsbW*, an antisigma factor that inhibits the stress-response regulator sigma factor B (36), 23 mutations in *clpX*, an ATP-dependent protease associated with degradation of misfolded proteins (37), and 20 mutations in *SAOUHSC_00670*, a putative low-affinity inorganic phosphate transporter gene (Fig. 3*A*).

Adaptation in alternating treatments occurs through mutational paths often differing from those of the single-drug treatments. We compared each gene's mutation frequency in each alternating treatment at day 22 with the sum of the mutation frequencies in the corresponding single-drug treatments at day 11 (to control for drug exposure time). We found one gene enriched for mutations in alternating treatments compared with single-drug treatments at a threshold of $P = 0.01$ (Fig. 3*B*): *rsbW,* acquired mutations in 14 of 20 TMP-CIP alternating treatment populations but in only one TMP-only population and in none of the CIP-only populations ($P = 0.0026$) (Fig. 3*B*). Similarly, three genes were depleted for mutations in alternating treatments (Fig. 3*C*; see Table S1 for a complete list of enriched and depleted genes). These findings show that genotypic evolution in the alternating-drug regimens cannot be modeled as simply the sum of mutations acquired in the single-drug treatments.

One type of deviation from the neutral genotypic expectation that corresponded with the observed rates of evolution was the relative number of mutations in the primary drug-target gene. Compared with CIP-only treatment, we find significantly fewer mutations in *grlA*, the most commonly mutated gene in that treatment, in the CIP-NEO treatments ($P = 0.0061$) (Fig. 3*C*), which are slower to gain CIP resistance (Fig. 2*B*). Similarly, mutations in *folA* are depleted in both TMP-NEO and TMP-CIP alternating treatments (by 50%, although not significant, $P = 0.1687$) (Dataset S2),

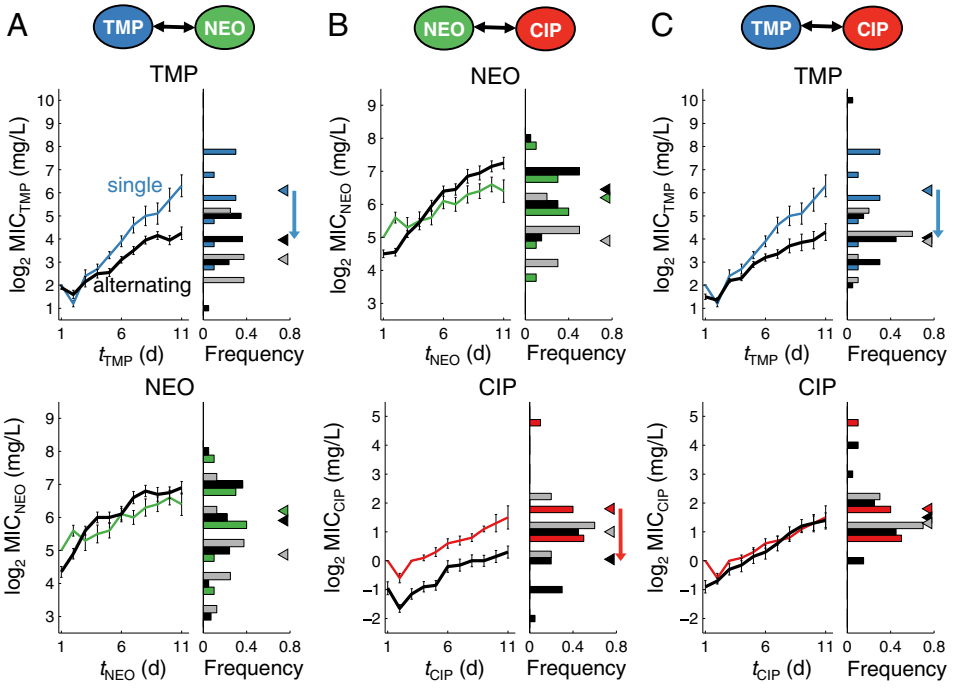


**Fig. 2.** Alternating drugs slows evolution of resistance to one of the two drugs. For each of the three drug pairs, (*A*) TMP-NEO, (*B*) NEO-CIP, and (*C*) TMP-CIP, the mean resistance (MIC) to each of the two drugs is shown both as a function of the time exposed to that drug (*Left*) and as a distribution at the final time point, 11 d of exposure to that drug (*Right*), for the single- (TMP in blue, NEO in green, and CIP in red), alternating- (black), and mixed-drug treatments (gray; resistance to single drugs measured at only final time point). Each line indicates the mean resistance of 10 single-drug or 20 alternating-drug independent populations, as inferred from the well chosen for propagation during evolution (individual trajectories vary) (Fig. S1). Error bars indicate SEM. Day 1 represents the resistance after 2 d of evolution for half of the alternating-drug populations, and may not match that of single-drug populations. Histograms reflect phenotypic measurements following 1 d of growth in the absence of drug (*Materials and Methods*) (may not match exactly with inferred resistance), and triangles indicate mean final phenotyped resistance. Downward-pointing colored arrows indicate statistically significant differences ($P < 0.05$, two-sample $t$ test) between the final resistance levels of the single and alternating drug treatments. In all drug pairs, resistance to one of the two drugs was slower in the alternating-drug treatment than in the single-drug treatment.

which are both slower to gain TMP resistance (Fig. 2 *A* and *C*). In contrast, the most common mutational target of NEO, *fusA*, is also abundantly mutated in alternating treatments (Fig. 3*A*), and the evolution of resistance to NEO is not impeded by alternating treatments (Fig. 2*B*). These data suggest that effective alternating treatments impede the acquisition of mutations in primary resistance genes.

Mixed- and alternating-drug treatments tended to select for similar sets of mutations (Fig. 3 *B* and *C*). In all genes with enrichment or depletion of mutations in alternating-drug treatments, the same trend was seen in mixed-drug treatments, although not always to statistically significant levels, partly because of the smaller sample size in mixed-drug treatments (Fig. 3 *B* and *C* and Dataset S2). Two genes were mutated at significantly different frequencies between the alternating and mixed treatment ($P < 0.01$) (Table S1): *fusA* (the main NEO resistance gene) was more frequently mutated in NEO-CIP alternating treatments than the mixed treatment ($P = 0.0015$), whereas mutations in *clpX* (likely conferring CIP resistance) were less frequent in the alternating treatments ($P = 0.0025$). These differences are consistent with the slower increase in NEO resistance and faster rate of CIP resistance in the mixed-drug treatment compared with the alternating drug treatment (Fig. 2*B*), and suggests that, compared with the alternating treatment, the selection imposed by mixed-drug treatment may have been biased toward CIP relative to NEO resistance. These data suggest that some mechanisms of evolutionary constraint are shared among multidrug treatments, whether alternating or mixed.

**Cross-Resistance Explains Evolutionary Constraint in Alternating Drugs.** We hypothesized that these phenotypic and genotypic differences between alternating- and single-drug populations might reflect constraints imposed by cross-resistance. Mutations with positive cross-resistance confer resistance to multiple drugs, whereas those with negative cross-resistance (i.e., collateral sensitivity) provide resistance to one drug but decrease resistance to another drug. Based on an earlier study of cross-resistance in *E. coli* (16), we predicted that alternating-drug treatments should be most effective in reducing the rate of resistance evolution when common resistance mutations exhibit negative cross-resistance.

To determine whether evolutionary constraint in alternating drugs is associated with negative cross-resistance, we selected, isolated, and profiled 40–44 single-step mutants resistant to each drug used in this study (Fig. 4*A*). To mirror the evolution experiment conditions as closely as possible when selecting these single-step mutants, we cultured wild-type cells in liquid media with inhibitory concentrations of antibiotic and plated on agar plates to isolate single colonies (*Materials and Methods*). Given the short culture time (20 h), we expect most of these isolates to have acquired only a single mutation. We measured the sensitivity of each single-step mutant to all three drugs, and found that, consistent with previous reports (16, 38–40), cross-resistance was frequent (69% of all mutants showed cross-resistance to at least one drug) and not always reciprocal. Sampling many mutants per drug revealed variation in cross-resistance, ranging from 2.8-fold resistance to 5.7-fold sensitivity in the same drug (Fig. 4*B*).

Despite this variation, the mean cross-resistance of single-step mutants selected in a drug correlated with whether or not alternating treatments slowed resistance to that drug in the evolution experiments. For example, most TMP-selected mutants showed negative cross-resistance to CIP but not vice versa (Fig. 4*B*). Correspondingly, populations evolved under alternating TMP-CIP treatment were less resistant to TMP, but not CIP, than those evolved in the respective single-drug treatments. This pattern suggests constraint on the acquisition of TMP-resistance, rather than the accumulation of negative cross-resistance mutations (which would predict slower evolution of resistance to CIP). Similarly, most TMP-selected mutants had negative NEO cross-resistance but NEO-selected mutants had no cross-resistance to the drugs used, as we would expect based on the patterns of resistance among populations evolved under alternating- and single-drug populations. We observe that the absence of cross-resistance for a given set of conditions correlates perfectly with the absence of change in the rate of resistance (three of three cases). Overall, the cross-resistance pattern of single-step mutants correctly predicted the resistance outcome of alternating treatments compared with single-drug treatments in five of six cases (all except CIP cross-resistance in NEO) (Fig. 4*C*).

In addition to explaining the overall resistance outcome of alternating treatments, cross-resistance may explain the enrichment

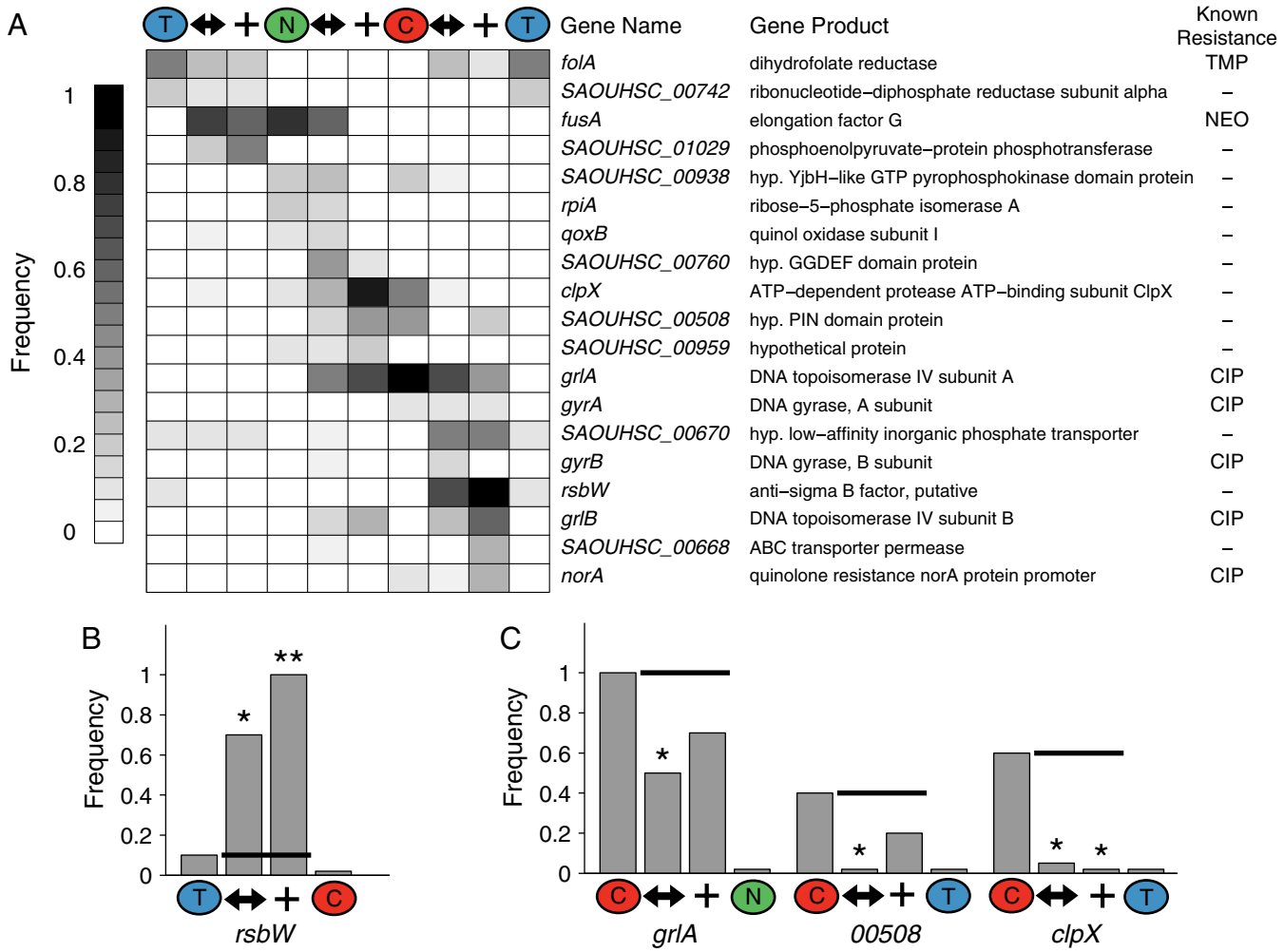


**Fig. 3.** Alternating drugs constrains evolution. (*A*) Genes with mutations in at least four populations are shown with their frequency in each of nine treatments: trimethoprim (T, shown twice), neomycin (N), ciprofloxacin (C), their pairwise alternating treatments (double-headed arrows), and mixed treatments (plus signs). Known resistance genes are indicated at the right. (*B*) Genes with significant enrichment ($P < 0.01$) for mutations in alternating treatment compared with single drugs. Black horizontal lines indicate expected mutation frequency in alternating treatment (sum of frequencies in single drugs), and statistical significance relative to this expectation was assessed with Fisher's exact test. *$P < 0.01$, **$P < 0.0001$. (*C*) Genes with significant depletion ($P < 0.01$) of mutations in alternating treatment. *$P < 0.01$. Genes with only locus tag names are indicated by the numerical portion of the locus name.

of specific mutations. We identified the only TMP-selected mutant showing positive CIP cross-resistance as a mutant in the gene *rsbW* by its orange pigmentation (see above for description; confirmed by sequencing) (*SI Materials and Methods*). As mentioned above, mutations in *rsbW* occurred frequently in the evolved populations treated with both TMP and CIP (both alternating and mixed), but only once among the populations treated with only one of the two drugs (Fig. 3*B*). In contrast, most single-step TMP mutants exhibited negative CIP cross-resistance, and would be selected against during exposure to CIP in an alternating treatment (Fig. 4*B*). Hence, although *rsbW* mutations result in low levels of TMP and CIP resistance, they are favored over other TMP-resistance mutations under alternating treatments, further suggesting that rapid alternation of drugs imposes evolutionary constraint via dual selection for resistance to both drugs.

## Discussion

We have presented phenotypic and genotypic analyses of the experimental evolution of *S. aureus* under single-, alternating-, and mixed-drug treatments. In all three tested drug pairs, alternating-drug treatments slowed the evolution of resistance to one of the two component drugs (Fig. 2). Using whole-genome sequencing, we found that alternating-drug treatments changed the spectrum of resistance mutations (Fig. 3). These genomic constraints and their impact on the rate of evolution are associated with cross-resistance among the drugs (Fig. 4).

Although cross-resistance profiles were strongly correlated with drug-resistance trajectories, cross-resistance is unlikely to be the only factor affecting evolution in alternating drugs. In addition to drug-specific cross-resistances, general fitness costs may slow evolution, even when a single antibiotic is alternated with periods of no drug. Another potential contributor is epistasis among resistance mutations within the same gene (41) and between genes (42). Epistatic mutations could alter the cross-resistance phenotypes of second-step and later mutations. Indeed, although most first-step TMP-resistance mutations conferred a cost in NEO, populations selected in only TMP did not have dramatically decreased resistance to NEO (Fig. S6). Such genetic interactions may explain why our measured CIP-positive cross-resistance in NEO did not correctly predict the rate of phenotypic evolution (Fig. 4*C*). Other factors might also affect the efficacy of such treatments, including competition (clonal interference) among resistant mutants, lasting nongenetic effects of drugs on bacterial physiology (43), effects of drugs on rate of mutations (44), and synergy and antagonism between the drugs (17). Additional experiments are needed to reveal these different mechanisms and understand how they

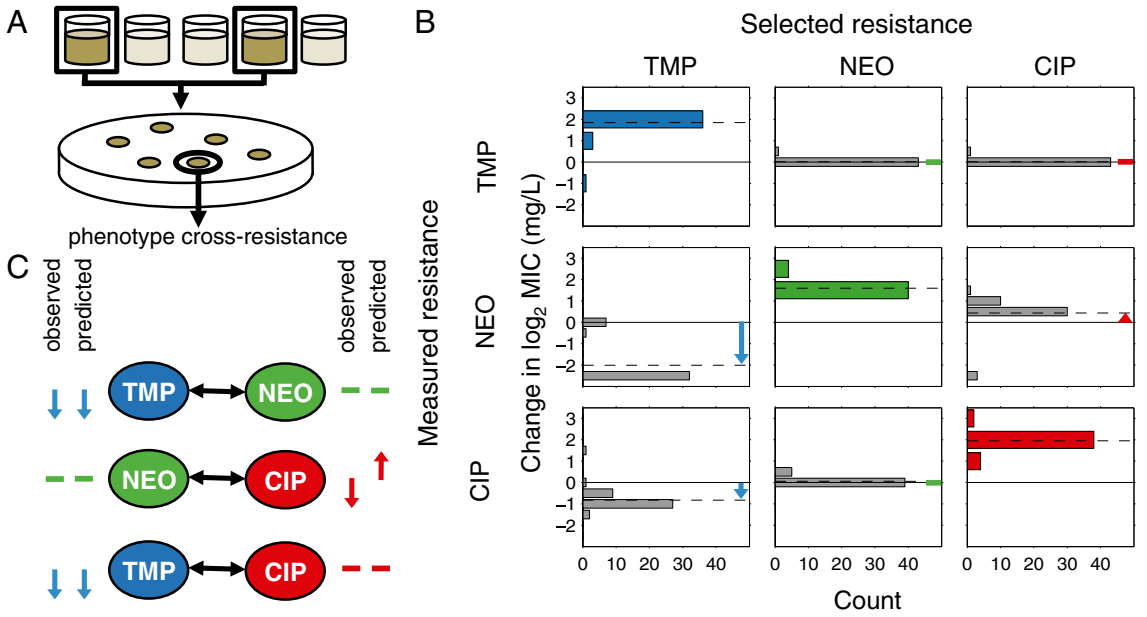


**Fig. 4.** Cross-resistance explains efficacy of alternating drug treatments. (*A*) Single-step mutants were generated by culturing wild-type cells in liquid media with antibiotic for 20 h, then isolated by pooling wells that grew to turbidity, and then plating these mutants on agar plates with enough antibiotic to prevent growth of the wild-type cells. At least 40 resistant colonies were selected for each drug and then phenotyped in all three drugs (*Materials and Methods*). (*B*) Cross-resistance of single-step TMP-, NEO-, and CIP-resistant mutants. Histograms show the distribution of resistance level (MIC) to the three drugs relative to the ancestor. The measurements of selected resistance are shown in colors and those of cross-resistance are shown in gray. Dashed lines indicate the mean resistance. Colored arrows indicate significant cross-resistance (mean change in $\log_2$ MIC > 0.25), and colored horizontal bars indicate no significant cross-resistance (mean change in $\log_2$ MIC < 0.25). (*C*) Cross-resistance correctly predicts effect of alternating drugs on both component drugs for the TMP-NEO and TMP-CIP drug pairs, and on NEO for the NEO-CIP drug pair (the only inconsistency is effect on CIP in NEO-CIP drug pair). Arrows indicate change in rate of evolution of resistance in alternating drugs compared with single-drug treatment, as observed during evolution experiments (Fig. 2) and as predicted by cross-resistance measurements (*B*). Horizontal bars indicate a prediction or observation of no change in rate of evolution of resistance.

combine to affect evolution of resistance in alternating- or even in mixed-drug treatments. Drug pharmacokinetics and dynamics also need to be considered to ensure their suitability for alternating therapy and to determine the most effective time scale for alternating drugs.

Despite these potential complicating factors, the correlation between slower evolution of resistance in alternating drugs and negative cross-resistance in our experiments suggests that negative cross-resistance may serve as a useful guide for the selection of drug combinations for alternating therapy in individual patients, as well as at the level of hospital wards. Ideally, further study might find drug combinations in which resistance to both drugs is impeded; these would be among the strongest candidates for use clinically. Although this study considered de novo mutations, we speculate that alternating therapy may also impede the horizontal acquisition of antibiotic resistance, because it increases the likelihood that bacteria with the acquired mechanism would be inhibited by at least one drug. Taken together, our findings suggest that appropriate drug combinations, either alternating or mixed, can limit the accumulation of negative cross-resistance mutations and impede the evolution of resistance.

## Materials and Methods

**Media, Strain, and Antibiotics.** All experiments were conducted in Luria broth (LB). The ancestral strain was *S. aureus* RN4220, a strain derived from NCTC 8325 but cured of phages (45). Aliquots of a culture inoculated with a single colony and grown overnight at 37 °C were stored in 16.7% (vol/vol) glycerol at −80 °C and used for all ancestral controls and to initiate evolution experiments. Stock antibiotic drug solutions were prepared from powder stocks and stored at −20 °C: TMP (Sigma-Aldrich T7883) at 50 mg/mL in dimethyl sulfoxide, NEO (Sigma-Aldrich N1876) at 50 mg/mL in $H_2O$, and CIP (Fluka 17850) at 10 mg/mL in 0.1N HCl. For mixed treatments, drugs were mixed at a fixed ratio of 4 TMP:16 NEO:1 CIP by mass, to obtain roughly equal inhibition of the ancestral strain from each drug.

**Experimental Evolution.** Evolution experiments were conducted in 96-well microtiter plates (Corning 3628) with a final volume of 160 μL per well. Samples were stored at −80 °C in a 1:6.67 dilution in 16.7% (vol/vol) glycerol between growth cycles. Because of the direct transfer from glycerol stocks into antibiotic solutions, evolving populations were cultured in media with ∼1% glycerol, and alternating treatments were exposed primarily to a single drug, but also a 1:100 dilution of the previous day's drug. See *SI Materials and Methods* for detailed description.

**Retrospective Phenotyping.** Before retrospective phenotyping, cultures were recovered by 100-fold dilution into media with 1% glycerol as in the evolution experiments but without antibiotic, grown overnight (same conditions as evolution), diluted 1:1.5 in 50% (vol/vol) glycerol, and stored at −80 °C. These "recovered" cultures were then used to inoculate fresh LB antibiotic solution 96-well plates with 150 μL per well, using a 96-pin tool (V&P VP407) that carries ∼1.5 μL (final dilution of ∼1:150). Inoculated microtiter plates were incubated for ∼20 h and then $OD_{600}$ was measured as in the evolution experiments. The lowest drug concentration at which $OD_{600}$ < 0.12 after background subtraction was considered the minimal inhibitory concentration (MIC). Three NEO-TMP samples and two TMP+NEO samples did not regrow during phenotypic measurements, and were omitted from analyses; however, phenotypic measurements correlated with MICs inferred during evolution experiments (Fig. S2).

**Phenotypic Analyses.** Statistical comparison of alternating- and single-drug treatments was done using the two-sample *t* test on retrospective phenotyping data, but using the two-sample Kolmogorov–Smirnov test resulted in similar *P* values and identical conclusions. Effect sizes were determined by interpolating the time required for the single-drug populations to acquire the same average level of resistance as the final resistance of the alternating populations, using the inferred resistance levels.

**Whole-Genome Sequencing.** A total of 121 samples were sequenced [available via National Center for Biotechnology Information Sequence Read Archive database, www.ncbi.nlm.nih.gov/sra (accession no. SRP045373, BioProject no. PRJNA257510)]: the ancestor, day 11 samples of all 10 populations of each of

the single-drug treatments, and day 22 samples of all 10 populations of each of the multidrug treatments. Half of the samples (the ancestor and TMP-CIP and NEO-CIP treatments) were sequenced using 100-bp paired-end reads on the Illumina HiSeq 2000 platform at the Massachusetts General Hospital NextGen Sequencing Core; the other half were sequenced using 101-bp paired-end reads on the Illumina HiSeq 2000 at Axeq Technologies. See *SI Materials and Methods* for detailed descriptions of library preparation and data-processing methods.

**Mutation Enrichment Analysis.** For statistical comparisons, mutations in intergenic regions immediately upstream of a given gene were considered to be in that gene. For comparing frequency of mutations in each gene across treatments, the number of populations with at least one mutation in a given gene was considered. Statistically significant enrichment and depletion in treatments was determined using Fisher's exact test. We do not directly account for multiple hypothesis testing but used a threshold of 0.01 and not 0.05 to minimize false-positives.

**Cross-Resistance.** Single-step resistant mutants were selected by liquid culture in antibiotic media followed by isolation via plating on agar plates. See *SI Materials and Methods* for detailed description of selection procedure. Then, 44 picked colonies were cultured overnight in LB with 1% glycerol, and phenotyped as described above for the evolution experiments, but with a 14-h incubation and $OD_{600}$ threshold of 0.06, and a finer gradient [sqrt (2)] for the drugs that were not used for mutant selection. Four TMP mutants that failed to regrow during phenotyping were omitted from analysis.

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