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# Collateral Resistance and Sensitivity Modulate Evolution of High-Level Resistance to Drug Combination Treatment in *Staphylococcus aureus*

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## Abstract

**As drug-resistant pathogens continue to emerge, combination therapy will increasingly be relied upon to treat infections and to help combat further development of multidrug resistance. At present a dichotomy exists between clinical practice, which favors therapeutically synergistic combinations, and the scientific model emerging from in vitro experimental work, which maintains that this interaction provides greater selective pressure toward resistance development than other interaction types. We sought to extend the current paradigm, based on work below or near minimum inhibitory concentration levels, to reflect drug concentrations more likely to be encountered during treatment. We performed a series of adaptive evolution experiments using *Staphylococcus aureus*. Interestingly, no relationship between drug interaction type and resistance evolution was found as resistance increased significantly beyond wild-type levels. All drug combinations, irrespective of interaction types, effectively limited resistance evolution compared with mono-treatment. Cross-resistance and collateral sensitivity were found to be important factors in the extent of resistance evolution toward a combination. Comparative genomic analyses revealed that resistance to drug combinations was mediated largely by mutations in the same genes as single-drug-evolved lineages highlighting the importance of the component drugs in determining the rate of resistance evolution. Results of this work suggest that the mechanisms of resistance to constituent drugs should be the focus of future resistance evolution work.**

***Key words:*** **resistance evolution, antibiotic resistance, drug combinations.**

## Introduction

Antibiotic resistance poses a severe threat to public health (Read et al. 2011; World Health Organization 2012). Left unresolved antibiotic resistance will increase the cost of healthcare, threaten medical advancement, scale back progress against certain infectious diseases and lead to greater morbidity and mortality (World Health Organization 2012). The increasing presence of antibiotic-resistant organisms has led to greater numbers of treatment failures for Gram-positive pathogens, such as methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, and multidrug-resistant tuberculosis (Cornaglia 2009; Woodford and Livermore 2009). The problem posed by resistant organisms is exacerbated by limited development of new antibiotics (Cottarel and Wierzbowski 2007; Fischbach 2011; Thaker et al. 2013). However, the arrival of new antibiotics provides only short-term relief as resistance quickly follows (Clatworthy et al. 2007; Read et al. 2011). Thus, the long-term key to controlling this threat lies in managing the unavoidable resistance adaptation (Read et al. 2011).

Combination therapy, the concurrent use of two or more drugs, is one such resistance management strategy, which has proven instrumental in prolonging the useful lifespan of antibiotics (Cottarel and Wierzbowski 2007; Read et al. 2011) as well as improving treatment outcomes in a variety of diseases, such as TB and HIV (Gilliam et al. 2006; Lennox et al. 2009; Huang et al. 2012; Vilchèze and Jacobs 2012; Freedberg et al. 2013). Combination therapy relies upon spontaneous resistance being rare and multiplicative so the likelihood of an organism gaining resistance to multiple drugs in a single instance is less than the prospect of resistance to any one of the component drugs acting alone (Fischbach 2011). This reasoning assumes that resistance acquisition is an independent event for each component of the mixture.

A major goal of resistance evolution research has been the search for the most effective yet resistance limiting combinations or treatment strategies (Yeh et al. 2006; Chait et al. 2007; Hegreness et al. 2008; Michel et al. 2008; Bollenbach et al. 2009; Torella et al. 2010; Imamovic and Sommer 2013; Pena-Miller et al. 2013). Outcomes of nearly a decade worth of experimental in vitro work have suggested that drug interactions (Chait et al. 2007; Hegreness et al. 2008; Michel et al. 2008; Torella et al. 2010; Palmer and Kishony 2013; Pena-Miller et al. 2013) are a key factor in limiting or driving resistance evolution, particularly during the early stages of resistance development. Specifically, combinations







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with antagonistic or suppressive interactions, where drugs in a mixture interfere with each other and the overall therapeutic effect is less than the component drugs working alone, have been shown to slow down resistance adaption better than those that act in a synergistic manner, where treatment outcomes are better than what would be expected from summing the effect of the component drugs acting alone (Chait et al. 2007; Hegreness et al. 2008; Michel et al. 2008; Torella et al. 2010; Pena-Miller et al. 2013). The rationale for this hypothesis is that the mutations conferring resistance to a single drug will have a more pronounced effect on the fitness of the organism in the presence of a synergistic combination because of the cooperative interaction of the components in the mixture (Hegreness et al. 2008; Michel et al. 2008). However, results of the in vitro work conflict with clinical practice where synergistic combinations are the preferred treatment regime (Cottarel and Wierzbowski 2007).

There are caveats to the paradigm that has emerged from these findings. These include the absence of the role of epistasis in driving resistance evolution (Trindade et al. 2009; Hall and MacLean 2011; Borrell et al. 2013) as well as the foundation being based on experimental work performed at or near WT minimum inhibitory concentration (MIC) levels (Yeh et al. 2006; Chait et al. 2007; Michel et al. 2008; Pena-Miller et al. 2013). Recent work has suggested that a better understanding of epistasis among relevant resistance conferring mutations could lead to the design of better treatment regimens (Trindade et al. 2009; Borrell et al. 2013). Moreover, clinically relevant resistance associated with treatment failures usually occurs in association with concentrations substantially greater than WT MIC levels (Anon 2013). Finally, emphasis on resistance adaptation at or near WT MIC levels may not accurately reflect the phenomena observed during the treatment of chronic bacterial infections, such as TB or cystic fibrosis. Despite the progress made through the aforementioned laboratory experiments, there is still a great need for a better understanding of the evolution of multidrug resistance (Palmer and Kishony 2013) before allowing these findings to shape or change therapeutic strategies aiming to control resistance evolution.

We proposed testing the generality of the current paradigm by extending the concentration range and adaptation time frame considered while using the same model organism and drug combinations originally used to construct it (Hegreness et al. 2008; Michel et al. 2008). We hypothesized that at elevated concentrations resistance evolution is driven by response to individual component drugs rather than drug interactions. To test our hypotheses, we evolved populations of *S. aureus* strain Newman, a medically relevant Gram-positive species, in the presence of six different antibiotics and five different combinations. The drugs and combinations used are well characterized, are clinically relevant, and have diverse modes of action (table 1). We performed genomic sequencing to determine the mutations involved in resistance adaptation. Finally, we considered the role of mutations in resistance toward drug combinations.

## Results

### Classification of Selected Drug Combinations

Drug combinations are characterized according to the epistatic interactions between their component drugs. The fractional inhibitory concentration index (FICI) is used to describe these interactions and is based on the Loewe additivity zero interaction theory (Berenbaum 1978). The index, determined for a given effect level, is the sum of the fractional inhibition of each drug in a combination relative to the drug acting alone. The interactions of each of our drug combinations were tested using the WT strain prior to commencing the resistance adaption experiments. The interaction types at an effect level of 90% were as follows: doxycycline–erythromycin (FICI $0.58 \pm 0.04$), doxycycline–ciprofloxacin (FICI $= 0.81 \pm 0.14$), and fusidic acid–erythromycin (FICI $= 0.75 \pm 0.15$) were synergistic, ciprofloxacin–ampicillin was additive (FICI $= 0.99 \pm 0.11$) and fusidic acid–amikacin was antagonistic (FICI $= 1.69 \pm 0.1$). Previous work performed in *Escherichia coli*, and performed again here (supplementary data S1, Supplementary Material online) characterized the interaction between doxycycline and ciprofloxacin as strongly antagonistic (Yeh et al. 2006; Toprak et al. 2011; Lázár et al. 2013); however, this combination was found to be synergistic when tested in our *S. aureus* strain Newman, underscoring the dependence of drug epistatic interactions on the specific target organism.

### Resistance Evolution of Populations

A wild-type (WT) *S. aureus* strain Newman population was challenged and adapted in three replicate lineages designated as A, B, and C to increasing concentrations of six individual antibiotics and five antibiotic combinations (table 1). An additional three replicate lineages, also designated as A, B and C, were passaged in media only. Adaptation was performed according to the following protocol (fig. 1). Briefly, the WT organism was inoculated into 12 different conditions with increasing concentrations of antibiotic(s) and allowed to grow for 18 h. At the end of the growth period, optical density (OD) measurements were taken and the most resistant culture from each replicate was reinoculated in fresh media at the drug concentration it was selected from. The recultured organisms were then used as inoculum for the next resistance challenge. A total of five resistance evolution periods, referred to as exposures, were performed. A total of ten inoculations (fresh media tube and exposure), equivalent to an average cumulative number of cell divisions (CCD) of $1.16 \times 10^{13}$ (Lee et al. 2011), were performed. A total of 36 lineages (18 single drug, 15 combination, and 3 media only evolved) were yielded through the evolution process.

Adaptation to single agents increased steadily with each exposure (fig. 2) for most populations and after five exposures four of six single-drug-evolved populations were able to grow in concentrations of at least 10 μg/ml (supplementary data S1, Supplementary Material online). Lineages evolved to erythromycin and amikacin developed resistance quickly and were able to grow in antibiotic concentrations greater

**Table 1.** Antibiotics Used and Their Modes of Action.

| Antibiotic Name | Abbreviation | Class | Target |
|---|---|---|---|
| Amikacin | AMI | Aminoglycoside | 30S ribosome |
| Ampicillin | AMP | Beta lactam | Cell wall |
| Ciprofloxacin | CPR | Quinolone | DNA synthesis |
| Erythromycin | ERY | Macrolide | 50S ribosome |
| Doxycycline | DOX | Tetracycline | 30S ribosome |
| Fusidic acid | FUS | Other | Protein synthesis |
| **Combination** | **Abbreviation** | **Interaction** | |
| Amikacin and fusidic acid | FUS-AMI | Antagonistic | |
| Ampicillin and ciprofloxacin | CPR-AMP | Additive | |
| Ciprofloxacin and doxycycline | DOX-CPR | Synergistic | |
| Erythromycin and doxycycline | DOX-ERY | Synergistic | |
| Erythromycin and fusidic acid | FUS-ERY | Synergistic | |

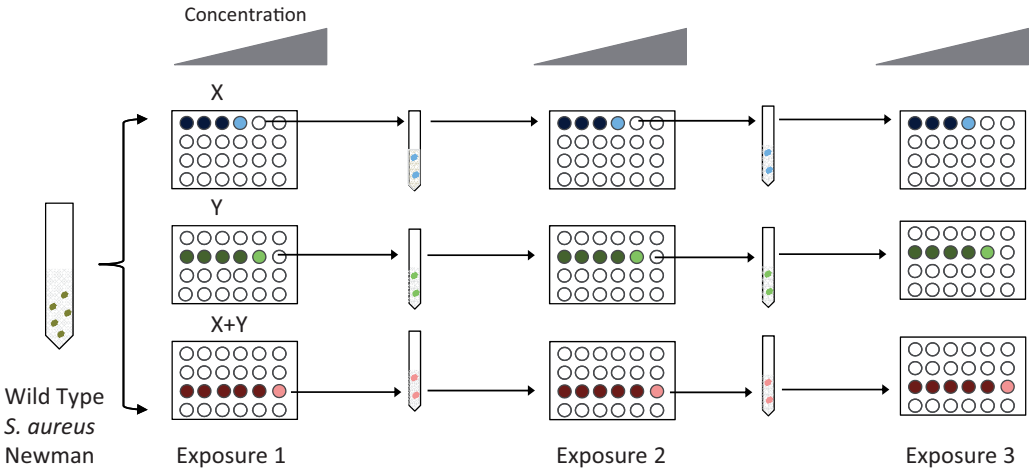


**Fig. 1.** Adaptation of *Staphylococcus aureus* to individual drugs and drug pairs. An overnight culture of WT *S. aureus* was used to inoculate microtiter plates containing different drugs or combinations with increasing concentrations or media only. Three replicate populations were recreated for each condition. The highest concentration where growth was present was recultured in fresh media and then used to inoculate the next concentration challenge, referred here to as exposure. A total of five exposures were performed for each condition.

than 100 μg/ml. Adaptation to doxycycline and ampicillin was much slower, with populations tolerating less than 3 μg/ml after five exposures. Adaptation by four of the five combination-evolved populations (ciprofloxacin–ampicillin, fusidic acid–amikacin, doxycycline–erythromycin, and doxycycline–ciprofloxacin) was similar to their slowest evolving single drug counterparts, whereas lineages evolved to the fusidic acid–erythromycin combination were approximately 10× less than their slowest evolving single drug counterpart (fig. 2 and supplementary data S1, Supplementary Material online).

## Resistance Profiles of Adapted Lineages

Following resistance adaptation, four isolates from each of the adapted populations were profiled for their individual resistances. Results show that all isolates exhibited a substantial increase in resistance following five exposures (fig. 3 and supplementary data S1, Supplementary Material online). In many cases, the IC90 values of the isolates were 100× greater than the WT value and in the case of the fusidic acid isolates more than a 1,000× larger. Exceptions to this trend were observed in the ampicillin, ciprofloxacin–ampicillin, and fusidic acid–erythromycin isolates where IC90 values were only 10–30× the WT value. Increased resistance differed among isolates evolved to the same drug(s) and in some cases this difference was considerable (fig. 3). We attributed the differences observed within a given drug(s) group to be the result of genotypic changes acquired by the isolates through adaption.

The fusidic acid–amikacin isolates (antagonistic interaction, supplementary data S1, Supplementary Material online) had the greatest increase in resistance improvement followed closely by isolates adapted to doxycycline–ciprofloxacin (synergistic interaction, supplementary data S1, Supplementary Material online). Isolates evolved to ciprofloxacin–ampicillin (additive interaction, supplementary data S1, Supplementary Material online) had the least resistance improvement, an average of 11× the WT MIC value. These results contrast with previous reports based on sub-MIC adaptations, which

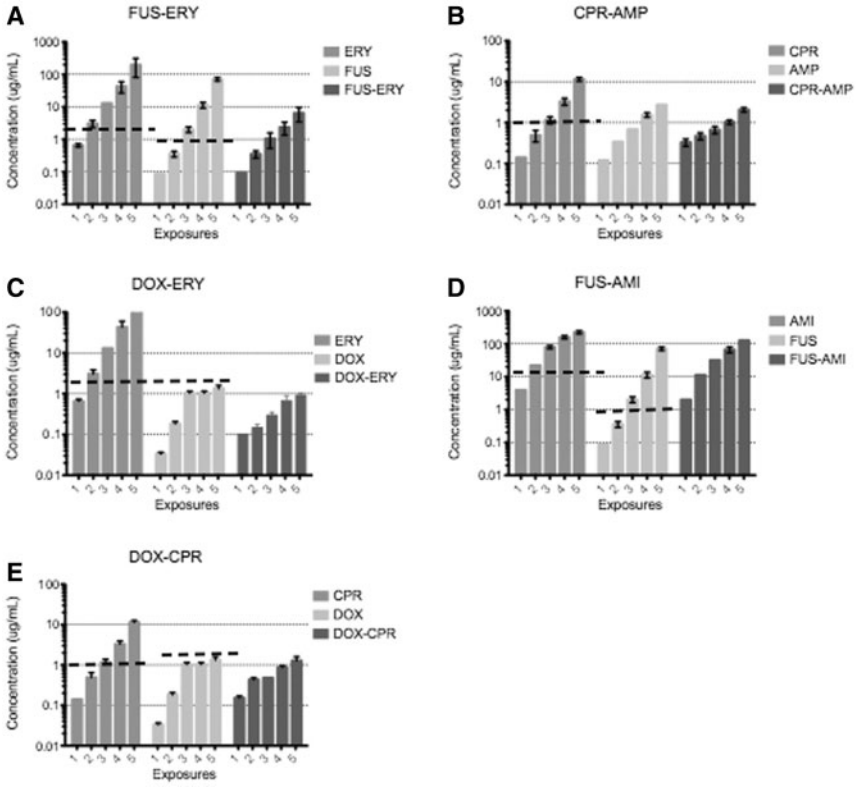


**Fig. 2.** Change in drug tolerance during adaptation. Each bar is an average of three replicate lineages and reflects the average concentration that the adapted population can grow in following exposure to ever increasing concentrations. Populations are grouped according to drug pairs: (*A*) FUS-ERY, (*B*) CPR-AMP, (*C*) DOX-ERY, (*D*) FUS-AMI, and (*E*) DOX-CPR. Dashed lines represent clinical breakpoints, taken from the EUCAST website, for each individual drug. There is no established clinical breakpoint value for ampicillin used on *Staphylococcus aureus*.

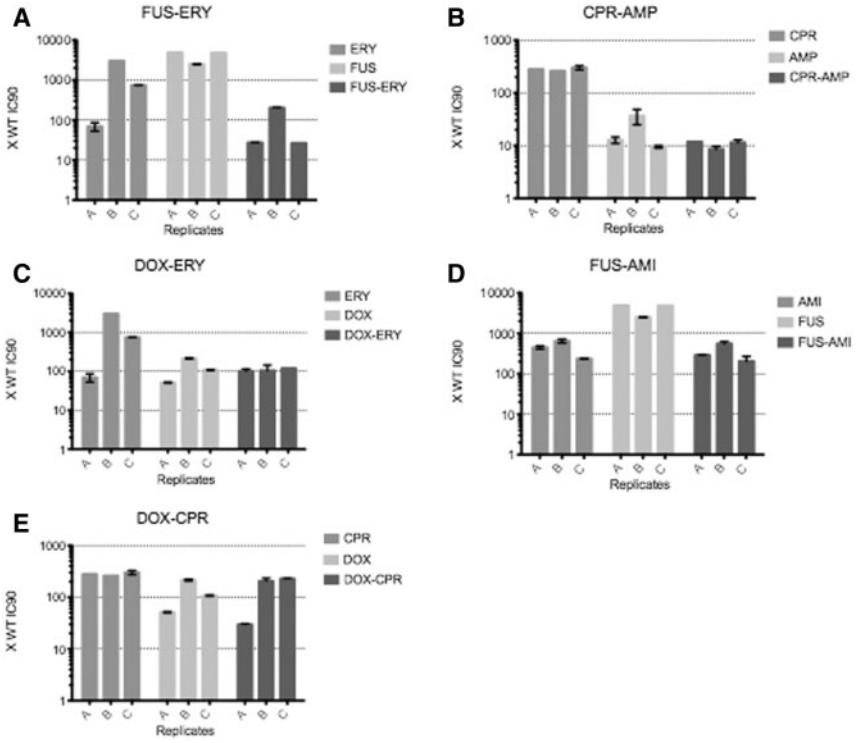


**Fig. 3.** Gain in IC90 value of the most evolved lineages following resistance adaptation. Isolates are grouped according to drug pairs: (*A*) FUS-ERY, (*B*) CPR-AMP, (*C*) DOX-ERY, (*D*) FUS-AMI, and (*E*) DOX-CPR. Each column is an average of four biological replicates. Error bars reflect the SEM of the replicates. Differences within a drug(s) group suggest that resistance adaptation is a complex process. Adaptation of the combination-evolved isolates mirrors that of the least evolved single drug isolates.

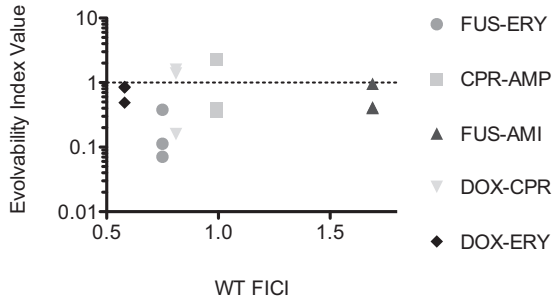


**Fig. 4.** Evolvability index values for each drug combination isolate. The evolvability index quantifies how being used in a combination impacted the resistance evolution to the individual component drugs of a combination. Values are grouped according to WT drug interaction. FUS-ERY, DOX-CPR, and DOX-ERY were all synergistic, CPR-AMP was additive, and FUS-AMI was antagonistic. Variation among replicates within the same drug pair reflects the individuality of resistance adaptation.

suggest that antagonistic or suppressive combinations limit resistance evolution best (Hegreness et al. 2008; Michel et al. 2008; Pena-Miller et al. 2013). In general, the extent of resistance attained by the combination-evolved isolates was similar to that of the slowest evolved corresponding single drug isolates, highlighting the importance of individual components in resistance evolution during combination therapy. We quantified these observations using the evolvability index (Munck et al. 2014), which describes how resistance evolution toward an individual drug is impacted as a result of being used in a combination compared with being used alone. The evolvability index is determined by taking the average of the relative change in resistance development for each component drug of a drug combination-evolved lineage and dividing it by the relative change in resistance development in the single-drug-evolved lineages (Munck et al. 2014) (eq. 2). An evolvability index value of 1 signifies that the combination-evolved isolate developed resistance to the same extent as the individual drug-evolved isolates did to the component drugs. A value greater than 1 indicates that the combination-evolved isolate evolved to be more resistant than its corresponding single-drug-evolved isolates, whereas a value of less than 1 means that the combination-evolved isolates evolved less than the single-drug-evolved isolates. It is important to note that the evolvability index assumes that the exposure time to each component or combination is the same. Comparisons where this is not the case are not accurate measures of resistance evolution. Nevertheless, this simplification provides a clear and quantitative means to compare how different combinations drive resistance adaptation across experiments and organisms.

All but three of our combination-evolved isolates had evolvability index values of less than 1 meaning that overall the combinations were effective at limiting resistance evolution relative to their constituent drugs alone (fig. 4 and supplementary data S1, Supplementary Material online). Isolates with evolvability index values greater than 1 were the ciprofloxacin–ampicillin isolate C (2.3) and the doxycycline–ciprofloxacin isolates B (1.38) and C (1.59). Each of these isolates had component IC90 values that greatly exceeded those of the corresponding single-drug-evolved isolates (supplementary fig. S1, Supplementary Material online). Elevated evolvability index values were also determined for fusidic acid isolate B (0.96) and doxycycline–erythromycin isolates B (0.86) and C (0.84) and were likely due to strong resistance to one component drug (supplementary fig. S1, Supplementary Material online). The smallest evolvability index values (<0.2) belonged to the fusidic acid–erythromycin isolates, which suggests that this combination limited resistance evolution best.

WT epistatic drug interactions were not found to be significantly correlated to the extent of resistance evolution observed. One explanation could be that drug interactions are not static but rather affected by resistance evolution. To assess the evolutionary stability of the epistatic drug interactions, we determined the FICI values for our combinations for the evolved isolates. These data show that changes in the drug interaction profiles had taken place (supplementary fig. S2, Supplementary Material online). For example, the interaction between doxycycline and ciprofloxacin postadaptation became antagonistic in each of the three replicate isolates. A similar shift was observed for two of the three fusidic acid–erythromycin isolates. The interaction between fusidic acid and erythromycin remained synergistic; however, the FICI values increased as a result of adaptation. An inconclusive interaction existed between ciprofloxacin and ampicillin following adaptation with one isolate demonstrating synergism whereas another displayed antagonism. FICI values for fusidic acid and amikacin decreased slightly below the WT value for two of the three isolates; however, the third isolate showed strong antagonism between the two drugs. These findings are in agreement with a recent study of *E. coli* exposed to erythromycin and doxycycline showing that drug interactions are strongly modulated by evolution (Pena-Miller et al. 2013). Drug interactions can predict resistance evolution for sub-MIC adaptation; however, our data suggest that these interactions change in response to resistance adaptation causing their reliability as resistance evolution predictors to become less certain.

Instead, we decided to investigate the role of cross-resistance in driving resistance evolution as there appeared to be a relationship between the resistance evolution of combination isolates and their corresponding constituent drug isolates. Moreover, cross-resistance has been suggested to play an important role in rates of adaptation (Szybalski 1954; Hegreness et al. 2008; Michel et al. 2008; Yeh et al. 2009; Imamovic and Sommer 2013; Lázár et al. 2013, 2014; Oz et al. 2014). Using the same combination pairings all single-drug-evolved isolates were exposed to the other respective component drug, that is, lineages evolved to drug A were exposed to drug B to test for cross-resistance in combination AB.

Overall, adaptation to a single antibiotic frequently resulted in the cross-resistance to another (fig. 5). The amikacin-evolved isolates had strong (>10× WT) cross-resistance to fusidic acid and in the case of one replicate the IC90 value was nearly 100 times that of the WT. Isolates evolved to ampicillin displayed limited to negligible cross-resistance or

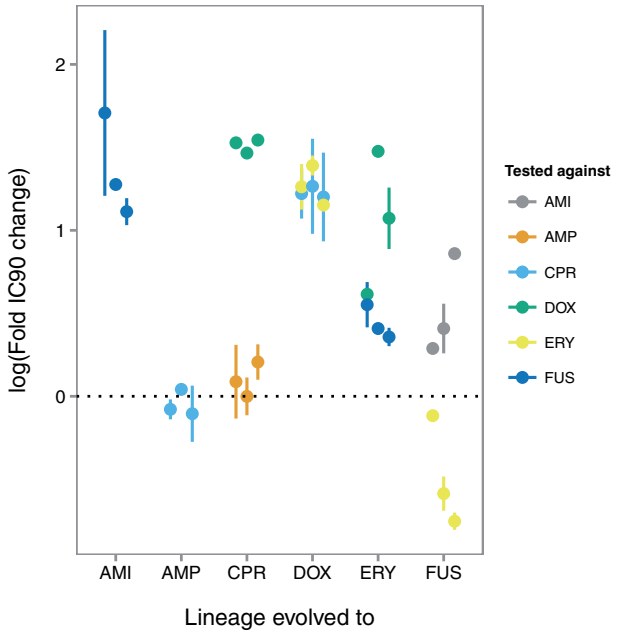


FIG. 5. Single-drug-evolved isolates tested for cross-resistance to their corresponding component drug. All single-drug-evolved isolates were tested to their corresponding component drug to test for cross-resistance or sensitivity. Each column is an average of four biological replicates and represents the gain or loss in WT IC90 value by drugs adapted to drug A tested against drug B. Error bars reflect the SD of the replicates. The isolates tested are listed below the *x* axis, whereas the drug they are tested against is given in the legend. Isolates evolved to fusidic acid displayed considerable sensitivity to erythromycin and moderate cross-resistance to amikacin. Isolates evolved to amikacin had strong cross-resistance to fusidic acid.

collateral sensitivity (Imamovic and Sommer 2013; Lázár et al. 2013) to ciprofloxacin and vice versa. The ciprofloxacin-evolved isolates, however, did display considerable (30× WT) cross-resistance to doxycycline. Adaptation to doxycycline resulted in strong (>10× WT IC90) cross-resistance to both erythromycin and ciprofloxacin. Isolates evolved to erythromycin displayed strong (>10× WT IC90) cross-resistance to doxycycline and moderate (<5× WT IC90) cross-resistance to fusidic acid. The extent of cross-resistance displayed by isolates evolved to ciprofloxacin, doxycycline, and erythromycin is consistent with the elevated evolvability indices calculated for the corresponding combinations. Finally, adaptation to fusidic acid resulted in collateral sensitivity to erythromycin with IC90 values well below the WT value. This collateral sensitivity likely explains the comparatively slow evolution of resistance observed for isolates evolved to the fusidic acid–erythromycin combination. The fusidic acid-evolved isolates also displayed moderate (<5× WT IC90) cross-resistance to amikacin. The combinations for which the component drugs did not confer collateral sensitivity exhibited significantly higher evolvability index values ($P < 0.05$, Mann–Whitney), suggesting that collateral sensitivity interactions between component drugs are important for determining resistance evolution toward drug combinations.

## Whole-Genome Sequence Analysis

To explore the molecular basis of the drug resistance observed in our experiments, we sequenced the genomes of our most evolved isolates (18 from the single-drug-evolved isolates, 15 from the combination-evolved isolates, and 3 from the media only-evolved isolates) and our ancestral WT. The sequenced isolates were then analyzed in groups based on the drug(s) they were evolved to. In general, the resistance phenotypes observed in the isolates could be readily explained by the presence of expected resistance mutations in their genomes.

An overlap of canonical resistance mutations was observed in both the combination-evolved and single-drug-evolved isolates (fig. 6 and supplementary data S2, Supplementary Material online). For example, two of three fusidic acid–erythromycin-evolved isolates (A and B) and two of three erythromycin-evolved isolates (A and B) had mutations in the *rplD* gene, which codes for ribosomal protein L4. Mutations in this gene have previously been associated with macrolide resistance in several bacterial species (Tait-Kamradt et al. 2000; Canu et al. 2002; Zaman et al. 2007), including *S. aureus* (Prunier et al. 2002). The mutations observed in the *rplD* gene of all four isolates are well-documented amino acid substitutions (Canu et al. 2002; Diner and Hayes 2009) that result in the alteration of the macrolide-binding site (Gregory and Dahlberg 1999; Gabashvili et al. 2001; Diner and Hayes 2009). The resistance conferred by these mutations, however, varied considerably (fig. 3 and supplementary fig. S1, Supplementary Material online) and appeared to be a function of quantity. Both erythromycin isolate B and fusidic acid–erythromycin isolate B had multiple single nucleotide polymorphism (SNPs) in the *rplD* gene, whereas erythromycin isolate A and fusidic acid–erythromycin isolate A each had only one SNP. Erythromycin isolate C had no ribosomal protein mutations but attained considerable resistance to erythromycin through an alternate means.

Mutations in the *fusA* gene, known to confer fusidic acid resistance in *S. aureus* (Besier et al. 2003), were observed in all isolates evolved to fusidic acid as well as the amikacin-evolved isolates. *fusA* gene mutations have previously been found to confer aminoglycoside resistance in *S. aureus* (Norström et al. 2007). The *fusA* gene mutations observed in the amikacin-evolved lineages conferred both high levels of amikacin and fusidic acid resistance, highlighting how cross-resistance can undermine the effect of drug combinations (figs. 2 and 5). It should be noted that fusidic acid and amikacin do not share overlapping binding sites. Fusidic acid binds to elongation factor G in complex with the ribosome (Turnidge and Collignon 1999), whereas amikacin binds to the 30S ribosome (Wright 2007).

The ciprofloxacin-, ampicillin-, and ciprofloxacin–ampicillin-evolved isolates shared a mix of well-documented canonical and lesser-known mutations. For example, all three isolates evolved to ampicillin and ciprofloxacin–ampicillin isolate B had mutations in the *pbpA* gene, which codes for penicillin-binding protein 1 (Wada and Watanabe 1998). Ciprofloxacin–ampicillin isolates A and C had mutations in an uncharacterized transport protein (NWMN600), which

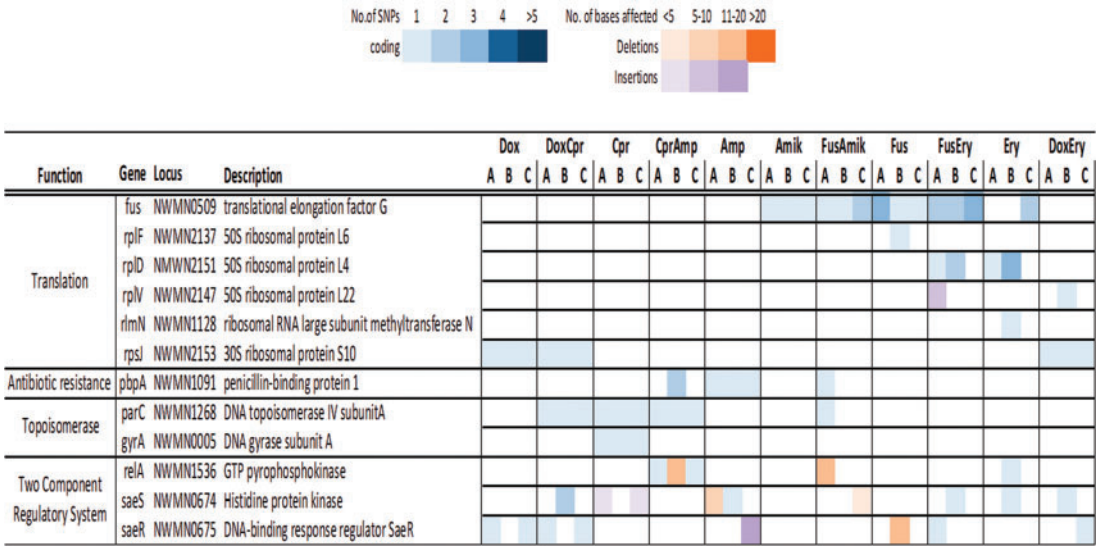


**Fig. 6.** Primary target genes affected by resistance adaptation. The most evolved isolates were sequenced and compared with the ancestral WT and the media adapted lineages to identify mutations resulting from resistance adaptation. Canonical resistance mutations were observed in both the single-drug- and combination-evolved isolates. Mutations associated with resistance to individual drugs dominated the mutations observed in the combination-evolved isolates.

may have helped provide resistance to ampicillin in the absence of mutations in penicillin-binding proteins (supplementary fig. S1, Supplementary Material online). Correspondingly, all isolates evolved to ciprofloxacin and ciprofloxacin–ampicillin had mutations in the *parC* gene, which codes for DNA topoisomerase IV subunit A, and is known to confer low-level resistance to ciprofloxacin (Janoir et al. 1996). The ciprofloxacin-evolved isolates had additional mutations in the *gyrA* gene, which is responsible for higher levels of quinolone resistance (Ferrero et al. 1995). When *parC* and *gyrA* mutations are both present an organism has high-level quinolone resistance (Janoir et al. 1996; Kaneko et al. 2000) (fig. 3). The deficiency of *gyrA* gene mutations manifested in the tolerance of ciprofloxacin by the ciprofloxacin–ampicillin-evolved lineages (supplementary fig. S1, Supplementary Material online). Reduced fitness was not observed for most of the isolates (supplementary data S1, Supplementary Material online).

All isolates evolved to doxycycline and its corresponding combinations, with the exception of one, had mutations in the *rpsJ* gene, which codes for the 30S ribosomal protein S10. Doxycycline targets the 30S ribosomal subunit and inhibits the binding of aminoacyl-transfer RNA (tRNA) to the mRNA ribosome complex. Ribosomal protein S10 is involved in the binding of tRNA to the ribsosome (Yaguchi et al. 1980) and mutations in this gene have previously been shown to confer high level tetracycline resistance in *Neisseria gonorrhoeae* (Hu et al. 2005). Doxycycline–ciprofloxacin isolate A was the only isolate without an *rpsJ* gene mutation. This isolate had the least resistance to doxycycline (supplementary fig. S1, Supplementary Material online) of all the doxycycline combination-evolved isolates. Moreover, the overall IC90 improvement by this isolate was 10× less than other two replicate isolates.

It should be noted that a variety of auxiliary mutations were observed in both the single-drug- and combination-drug-evolved isolates and appear to support the principal target mutations. These supplementary mutations were assessed and grouped according to function (supplementary data S2, Supplementary Material online). Instances of shared auxiliary mutations between the single-drug- and combination-evolved isolates were limited; however, the numerical distribution of these mutations was approximately equal among all sequenced isolates. Many of the auxiliary mutations were part of a larger stress response network, which likely participated in or aided resistance. For example, all isolates evolved to ciprofloxacin–ampicillin had mutations in the *relA* gene, which initiates the stringent response under environmental stress. This controls the production of the alarmone ppGpp, which in turn serves as a regulator of a variety of metabolic pathways and processes and has been shown to play an essential role in decreased sensitivity to penicillin (Kusser and Ishiguro 1985, 1987; Rodionov and Ishiguro 1995; Wu et al. 2010) and quinolones (Viducic et al. 2006). *relA* mutations were also observed in fusidic acid–amikacin isolate A and erythromycin isolate B.

In spite of the auxiliary mutations observed in the evolved strains, mutations associated with resistance to individual drugs dominated the mutations found in the combination-evolved isolates. Speed of resistance development by combination-evolved lineages was a function of how these mutations interacted to cause either cross-resistance or cross-sensitivity. In the case of the doxycycline–ciprofloxacin- and doxycycline–erythromycin-evolved isolates, the mutations required for resistance to the constituent drugs resulted in considerable cross-resistance between the single-drug-evolved isolates and culminating in elevated evolvability values for the combination-evolved isolates. A similar

situation was observed for the fusidic acid–amikacin-evolved isolates, where the same single resistance mutation was required for both constituent drugs resulting in cross-resistance between the single-drug-evolved isolates. In contrast, adaptation to fusidic acid and erythromycin resulted in strong cross sensitivity and was reflected in the reduced evolvability values of the combination-evolved isolates. Our findings stress the importance of collateral effects in limiting resistance evolution and not drug interactions.

## Discussion

We sought to extend the current scientific paradigm by expanding the concentration ranges considered to envelope concentrations likely to be encountered during clinical treatment. The motivation for this pursuit stems from the fact that treatment failure typically occurs at elevated concentrations. We pursued our study using the same drugs, combinations, and organism previously employed to develop the existing model for predicting resistance evolution based on drug interactions. We hypothesized that at concentrations above WT MIC, resistance evolution to drug combinations would be driven by the constituent drugs and collateral sensitivity interactions.

We were unable to reproduce the expected correlation between resistance evolution, as measured by evolvability, and drug interactions, as assessed by the fractional inhibitory combination index, at drug concentrations above WT MIC. We hypothesize that this is due to the fact that drug interactions are modulated by resistance evolution. The dynamic nature of drug interactions challenges their use as reliable predictors of long-term resistance evolution.

Results of our experimental evolution and genome sequencing work suggest that the evolutionary responses to individual constituent drugs are better predictors of resistance evolution. A drug pair where adaptation to one constituent drug confers cross-resistance to the other or where both constituent drugs share the same resistance mutations will undermine the effect of the combination and will likely have greater resistance evolution due to cross-resistance. In contrast, a pair where resistance evolution to one constituent results in collateral sensitivity to the other will have slower or reduced evolution due to the incompatibility of the individual resistance profiles. Finally, in between these two poles is the case where resistance to constituent drugs is unrelated/independent. Resistance to this drug pair is achieved in a measured fashion by individually acquiring mutations for each of component drugs.

In conclusion, we find that above WT MIC levels, individual constituent drugs and their associated resistance mutations are reliable predictors of a combination's potential resistance evolution. Mutations associated with resistance to one constituent drug of a combination have the power to either promote or obstruct resistance to another component in the same combination. We suggest that rather than continuing to focus on drug interactions, further research should consider the mutations that will arise from resistance adaptation and pursue those combinations with diverging evolutionary trajectories, as these combinations will likely limit resistance evolution best.

## Materials and Methods

### Bacteria and Reagents

A drug sensitive *S. aureus* strain Newman was adapted to five antibiotics: Amikacin sulfate (Sigma), ampicillin sodium salt (Sigma), ciprofloxacin hydrochloride (AppliChem), erythromycin (Sigma), fusidic acid sodium salt (Sigma), and doxycycline hyclate (TCI) and the following drug pair combinations: fusidic acid–amikacin, fusidic acid–erythromycin, ampicillin–ciprofloxacin, doxycycline–ciprofloxacin, and doxycycline–erythromycin. Drug stock solutions were prepared weekly. All evolution and MIC experiments were performed using a modified Luria broth (LB) media. The salt content was reduced to 4 g/l instead of 5 g/l.

### Evolution of Antibiotic Resistance

A WT IC90 was established for each antibiotic. Drug pair combinations were a 1:1 IC90 mixture of the component drugs. WT IC90s were also established for each drug pair. All evolution experiments began one dilution step below their respective IC90 concentration. Evolution experiments involved challenging a WT organism with increasing concentrations, in steps of the square root of 2, of individual drugs or drug combinations. All evolution experiments were performed in triplicate in a modified Luria–Bertani (LB) broth in microtiter plates. Each experiment included both negative and positive control wells. The positive control was the inoculating strain in LB media only. Following an 18-h growth period at 37 °C, the microtiter plates were measured for OD at wavelength 600 nm (OD600). The value of the experimental positive control was used to normalize the evolution data. A cut off of 60% inhibition was used to determine the starting concentration of the next experiment. This concentration was referred to as the experimental MIC. The 60% inhibition value was chosen based on pre-experimental work that found that this value consistently ensured a resistant population was used in subsequent exposure experiments. The replicate with the best growth at the experimental MIC concentration was used as seed material for the next experiment. The selected seed was added to fresh LB media containing the appropriate drug(s) concentration and allowed to grow over night. The overnight culture was then used to inoculate the next challenge experiment. A portion of this culture was saved. The challenge process was repeated a total of five times for each individual drug and drug combination. The same adaptation procedure was used for the media only evolved populations.

### IC90 Determination

Following adaptation, isolates of the adapted populations were profiled for their individual resistances. IC90 determination was performed according to standardized methods (Andrews 2001). Briefly, lineages from the fifth exposure were plated on nonselective media and allowed to grow overnight. Four individual isolates were then randomly selected

from each plate and grown in nonselective liquid media for 4–5 h before being used to inoculate additional IC90 experiments. All single-drug isolates were tested against the agent they had been adapted to as well as their corresponding drug combination and matching component drug. Combination-evolved isolates were tested against the combination to which they had been adapted and the commensurate component drugs. In both the population and single isolate experiments, the inoculum size for each well was approximately $10^4$ cells. All IC90 experiments were performed in 96-well microtiter plates in quadruplicate using 2-fold dilution steps. Positive, isolate in LB media only, and negative controls were included in each test. Inoculated plates were placed on an orbital shaker (300 rpm) and incubated at 37 °C for at least 16 h. After the allotted growth period, OD600 was read on a BioTek Epoch plate reader.

## Calculation of CCD

Using the equation set forth by Lee et al (2011), $n$ is the number of generations for each growth step. In our case, there are two growth steps—the resistance experiment and the test tube pregrowth period prior to each resistance experiment. $n$ values were calculated for each evolved lineage and the two growth steps.

We performed growth kinetic experiments that allowed us to calculate a generation time ($G$ in $\text{min}^{-1}$) for each strain. These values were then used to determine the number of generations for each strain in an 8-h period (assumed log growth phase) or $n$.

In the Lee equation, CCD is

$$\sum_{I=1}^{M} N_0(2^N - 1), \qquad (1)$$

where $N_0$ is the initial number of cells in each well or test tube during evolution. We used representative values of $N_0$, reflecting each growth condition, for each strain to calculate the CCD for the test tube and resistance experiment periods. The subsequent CCD values were multiplied by 5 to reflect the number of evolution periods for each growth condition. A CCD value was calculated for each replicate lineage (supplementary data S1, Supplementary Material online). The average CCD value in the text comes from adding the two growth conditions together.

## Data Analysis

The OD600 data were analyzed using Excel and Prism (GraphPad Software). Briefly, negative control values were subtracted from all growth wells yielding dose–response values. These data were then normalized by the positive control data and then used to determine the fraction of inhibition, calculated as: 1 − normalized dose response of strain X. Inhibition data were plotted in Prism and IC90 read from graph.

## Calculation of Evolvability Index

The evolvability index assesses how resistance evolution toward a combination compares with individual drug resistance evolution. The index is determined by summing a combination-evolved strain's resistance to each of its component drugs relative to the resistance development of the corresponding single-drug-evolved lineages and then taking an average. Each individual fraction can be used to assess how resistance evolution to an individual component is impacted as a result of being used in a combination. The evolvability index is calculated as:

$$\text{Evolvability Index} = \frac{1}{n}\left\{\frac{\text{IC90[A]}_{\text{AB}}}{\text{IC90[A]}_{\text{A}}} + \frac{\text{IC90[B]}_{\text{AB}}}{\text{IC90[B]}_{\text{B}}}\right\}, \qquad (2)$$

where the $n$ is the number of components in a mixture and is used to determine an average value. $\text{IC90[A]}_{\text{AB}}$ refers to the IC90 of the AB-evolved lineage tested against drug A.

## Sequencing

Genomic DNA from our most evolved strains and WT was isolated using either an UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Inc.) or a modified chloroform/phenol extraction method. Briefly, lysostaphin in conjunction with proteinase K was used to disrupt the cell wall. The extracted DNA was sheared into 200-bp fragments using a Covaris E210 and barcoded libraries were constructed for Illumina or IonTorrent sequencing. Illumina sequencing was performed by Partners HealthCare Center for Personalized Genetic Medicine (Cambridge, Massachusetts) and by Sequencing, Informatics and Modeling Group at The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark (Hørsholm, Denmark). IonTorrent sequencing was performed by DTU Multi-Assay Core (Kongens Lyngby, Denmark). All reads were aligned to *S. aureus* subsp. *aureus* str. Newman (NC_009641.1) using Bowtie2 version 2.0.0-b6 with the default options (Langmead and Salzberg 2012). An average of 99.6% (minimum 97.5%) of the genome was covered with an average read coverage of $125 \pm 40$ (CI95) (supplementary data S2, Supplementary Material online), as determined using BEDTools (Quinlan and Hall 2010). Variant calling for SNPs and INDELs was done using SAMTools version 0.1.17 with the –B,-L 1,000 options(Li et al. 2009). Only SNPs with a phred score of at least 30 and where at least 80% of the reads aligned at the site had the variant were used. INDELs were verified by aligning constructed contigs around INDEL sites to the reference genome (Zerbino and Birney 2008; Li and Durbin 2009). The BioCyc database collection (Karp et al. 2005) was used to identify and annotate mutation sites.

# Supplementary Material

Supplementary data S1 and S2 and figures S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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