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Cross-Resistance, Collateral Sensitivity, Antibiotic Interactions, and Their Influence on Antibiotic Resistance Evolution in Bacteria

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biology

by

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ABSTRACT OF THE DISSERTATION

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Professor Pamela Yeh, Chair

The prevalence and strength of multi-drug antibiotic resistance have resulted in an arms race between the development of new treatment options and the evolution of resistance in bacteria. The combination of drug therapies and antibiotic cycling presents possible solutions to this problem. However, these solutions introduce new factors to consider (see Chapter 1). This dissertation uses experimental evolution to broaden our understanding of resistance evolution to multiple antibiotics used in combination. It was found that the range of antibiotic concentrations that can select for resistant mutants widens once resistance has evolved (Chapter Two). In addition, this work investigated how the genetic background of resistant strains affects the viability of four effective 3-drug combinations and each of the individual drugs that make up the combination (Chapter Three). This work also evaluated the presence and persistence of an understudied type of drug interaction, hidden suppression, in 3-, 4-, and 5- drug combinations (Chapter Four). Hidden

suppression occurs when the combined effects of multiple antibiotics result in more bacterial growth than the effects of a smaller subset of those same antibiotics. Finally, this work asked if the drug interactions within a 3-drug combination can affect the rate at which resistance evolves in *Staphylococcus epidermidis* (Chapter Five). Using antibiotic resistance as a model system not only helps to fill the knowledge gap to solve a public health crisis but also allows me to address fundamental questions in evolutionary biology. For example, this work directly addresses how a combination of stressors affects the evolution of an entire population with varying genetic backgrounds. Overall, this research integrates this evolutionary perspective to determine how different antibiotic treatments affect the adaptation rates, adaptation frequencies, and resistance strengths of bacterial populations.

The dissertation of Natalie Ann Lozano-Huntelman is approved.

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This dissertation is dedicated to both my grandfathers, Juan C. Lozano and Wilburn ‘Ray’ Watson, who encouraged and supported all aspects of my education.

To my family and husband, without you I would not have achieved this dream.

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Natalie Lozano, Ashley J.R. Carter, "Selection Bias and Modularity's Effect on Evolvability Through Individual-Based Simulations", College of Natural Sciences and Mathematics Symposium, California State University, Long Beach, Long Beach, CA, USA, poster 43 (Sep. 2016)

Awards and Honors

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The Schmidt Science Fellows, in partnership with the Rhodes Trust, seeks to create a new generation of scientific leaders. The program provides awards to the brightest minds in STEM and computing to broaden your perspective, networks, and skills. UCLA is allotted four nominees per year.

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This program is intended to support doctoral students who are advanced to candidacy at the time of nomination by their department to the Graduate Division. Program participants will receive a $20,000 stipend plus standard tuition and fees for three quarters. Program to begin either Summer, Fall, or Winter quarter.

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Departmental Research Award – UCLA (EEB) **Spring 2020**

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The program provides awards to pairs of dissertation advisers and their graduate students based on what HHMI values and considers essential components of the environment, particularly the institution and adviser's commitment to advance diversity and inclusion in the sciences and the student's potential for scientific leadership.

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Year: 1 & 2

Dean's Funds Award – UCLA **Fall 2017**

This award is granted to one incoming student in recognition of scholarly achievement. The fellowship provides $15,000 of support during your first year.

National Institute of Health's RISE MS to Ph.D. Scholar - CSULB **2015- 2017**

Fellows receive partial tuition support and an annual stipend of roughly $19,200. The program also provides support for some research supplies and travel to present research results at professional conferences.

Chapter 1: Background and Significance

Antibiotic resistance is a growing problem facing humanity on a global scale (Neu, 1992; Roca et al., 2015; Ventola, 2015; Blair, 2018; MacFadden et al., 2018; Povolo & Ackermann, 2019). It has been attributed partly to the misuse and overuse of antibiotics. This allows for bacterial populations to evolve resistance (Ventola, 2015) creating an arms race between the development of new antibiotics and the evolution of resistance. More bacteria are gaining single and multi-drug resistance (Bush et al., 2011; Spellberg & Gilbert, 2014). Antibiotic resistance requires new disease management strategies and is an added burden of healthcare costs (Tenover, 2006). Taking an evolutionary perspective to antibiotic resistance offers a unique understanding of how different treatments affect the adaptation rates, frequencies, and resistance strengths of bacterial populations. With this information the evolutionary rates and trajectories can potentially be manipulated to lead to less resistant or more sensitive bacterial population.

Antibiotics are responsible for killing or inhibiting bacterial growth in low concentrations (Waksman, 1947). They can be categorized in several different ways based on the chemical structure, the organisms that produce them, and the main mechanism that they affect (Russell, 1998; Wright, 2005; P. Yeh et al., 2006). This work will focus on categorizing antibiotics by the main mechanism. The main mechanism of an antibiotic can show the general target of the antibiotic indicating what aspects of the cell's phenotype selection is acting on. There are five main mechanistic categories: cell wall inhibition, inhibition of protein synthesis, alteration of cell membranes, inhibition of nucleic acid synthesis, and antimetabolite synthesis. These five categories can then be further broken down into subcategories. For example, cell wall inhibition can be achieved multiple ways: beta-lactams inhibit peptidoglycan synthesis, vancomycin disrupts cross-linkages between peptidoglycans, bacitracin disrupts movement of peptidoglycan

precursors, and antimycobacterial agents disrupt mycolic acid or arabino glycan synthesis (Hancock, 2005).

When a bacterial population is treated with a single antibiotic, one of three things can occur: 1) the concentration of the antibiotic can be so low that it does not affect the growth of the cells, 2) the concentration of antibiotic is so high all cells are killed, or 3) the concentration of antibiotic is high enough to kill some of the population but not all of it, allowing for resistant mutants to be selected for. Finding the cutoff concentrations that lead to each of these outcomes is achieved through the determination of the minimum inhibitory concentration (MIC) and the mutant prevention concentration (MPC). The MIC is the lowest concentration of an antimicrobial agent that limits visible growth. There are multiple ways to determine MIC. These include using liquid media in culture tubes or well-plates, or on agar plates using E-test strips or disk diffusion (Reller et al., 2009). Comparing the MIC of a mutated strain to its ancestral strain quantifies the amount of resistance or sensitivity gained by the mutation(s). A resistant strain will have a higher MIC than then the MIC of the wild-type or ancestral strain. A sensitive strain should have a lower MIC than the ancestral strain. The MPC is the concentration that requires at least two concurrent point mutations to achieve growth (Blondeau et al., 2004). The range between the MIC and MPC indicates the mutant selection window that allows for strains to evolve resistance (Drlica & Zhao, 2007).

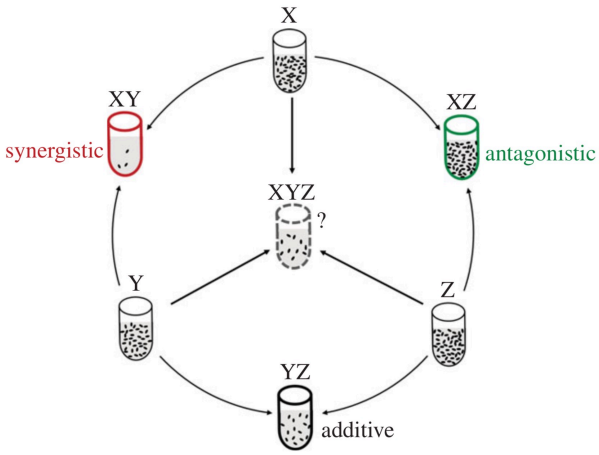

Figure 1-1. Drug Interactions Schematic (from Tekin, et al., 2017)

Bacteria can have antibiotic resistance primarily through one of these two ways: 1) acquired resistance through spontaneous mutation and 2) acquired resistance through horizontal gene transfer (Blair et al., 2015). For example, cells may acquire a mutation altering penicillin-binding protein 2b in pneumococci, which results in penicillin (a beta-lactam) resistance (Tenover, 2006). However, when multiple antibiotics are used in combination or in a sequence, it leads to new aspects to consider. Now, the cell is no longer only facing one specific mechanism to develop resistance to but rather multiple mechanisms all at once. This can be challenging, for an individual to gain resistance, a single point mutation must affect at least two or more different structures or physiological pathways.

Antibiotic combinations can be categorized by the types of interactions that result from the combined effect of each individual antibiotic component, Figure 1-1. A combination is considered additive if the combination yields the expected response of the combined effects based on the single drugs alone. A synergistic combination yields a stronger response than that additivity. These combinations have a stronger selection strength, making it more likely for a population to purify resistance mutations within the population. This increase in selection pressure causes beneficial mutations to sweep through a population quickly (Orr, 2000; Pepin & Wichman, 2008). Finally, an antagonistic

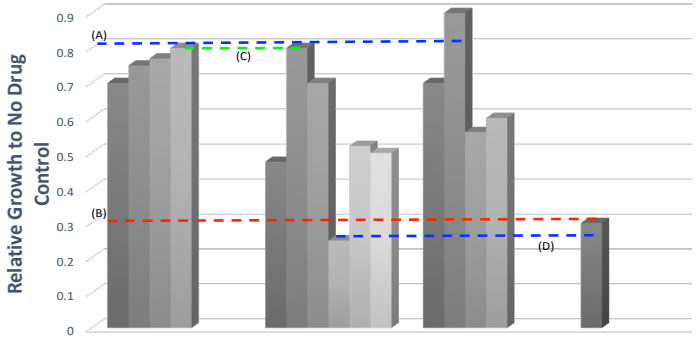

Figure 1-2. The differences among antagonistic interactions. Line A and D (blue dashed line) show instances of suppression. Line A shows a suppression in one of the 3-drug combinations with higher growth than any of the single antibiotics. Line B (red dashed line) shows how compared to the single drugs a 4-drug combination can be considered additive or even synergistic. But Line D shows that when compared to one of the 2-drug combinations it is in fact a hidden suppressive combination. Line C (green dashed line) shows an example of antagonistic buffering.

combination gives a weaker response when compared to the additive effects. There are two special cases of antagonism, buffering and suppression (Figure 1-2). Buffering is when the effects of a drug combination is equal to the highest lower-order or single drug effects (Tekin et al., 2017). The most extreme form of antagonism is suppression, it occur when the combined stressors together work less well than a single or lower order combination of stressors on their own (P. Yeh et al., 2006; Tekin et al., 2018). Antagonistic combinations are typically avoided in the clinic because they can require larger doses to have the same killing efficiency as synergistic combinations, but have the advantage of slowing the evolution of resistant strains (P. J. Yeh et al., 2009).

Bliss independence (Bliss, 1939) is used to quantify additive, synergistic, and antagonistic interactions (Beppler et al., 2016; Tekin et al., 2016; Beppler et al., 2017; Tekin et al., 2017). Bliss independence assumes that the relative effect of each antibiotic at a set concentration are independent of each other and is therefore is used to define additivity. Positive or negative deviations from this additivity are then considered to be antagonistic or synergistic, respectfully. For example, if two antibiotics, antibiotic X and antibiotic Y, each inhibit growth by 50% alone the combination of antibiotics XY would result in the expected inhibition of growth would be 75% ($0.75 = 1 - 0.5 \times 0.5$) (P. J. Yeh et al., 2009). If the expected inhibition of growth was observed when antibiotics XY were used in combination the interaction would be considered additive.

In a multi-drug combination there are many factors that contribute to the overall fitness effect of the combination. The first factors to consider are the additive effects from each drug alone. The next set of factors are the effects of each of the smaller sub-sets of interactions that interact additively with the other antibiotics (or combination of antibiotics) in the mix. The final

factor is the highest order emergent effect, the effect of the interaction among all drugs present in the combination. All of these factors are used to describe the interactions of a three-drug combination. These descriptors are the overall effect, the net deviation from additivity (DA), and the emergent effect. The net deviation from additivity, DA, is determined by only removing the fitness effects contributed by each drug alone from the overall fitness effect assuming Bliss independence. Once the net DA is determined the process can be done again, removing not only the additive contributions of each drug but also the effects of all lower order interactions leaving the emergent effect (Beppler et al., 2016). This framework is used to examine two-, three-, four-, and five-drug combinations but can also be expanded to *N* number of drugs (Tekin et al., 2018). To find the net deviation from additivity for N drugs (DA_N) we remove the additive effects of each individual drug (Equation 1). To find the higher order emergent we now remove all the lower order interactions from the DA leaving the value of the highest order interaction possible. Equation 2, shows an example calculation for the emergent interaction E_4 of a four-drug combination.

Equation 1: $$[DA_N]_{D_1,D_2,D_3\ldots D_N} = w_{D_1,D_2,D_3\ldots D_N} - w_{D_1}w_{D_2}w_{D_3}\ldots w_{D_N}$$

Equation 2: $$E4 = w_{w,x,y,z} - w_w w_{x,y,z} - w_x w_{w,y,z} - w_y w_{w,x,z} - w_z w_{w,x,y} - w_{w,x}w_{y,z} - w_{w,y}w_{x,z} - w_{w,x}w_{x,y} + 2w_w w_x w_{y,z} + 2w_w w_y w_{x,z} + 2w_x w_y w_{w,z} + 2w_x w_z w_{w,y} + 2w_y w_z w_{w,x} - 6w_w w_x w_y w_z$$

Antibiotic interactions can influence the evolution of antibiotic resistance within populations. When examining two-drug interactions, Michel et al. (2008) show that synergistic drug combinations promote the evolution of antibiotic resistance. Synergistic interactions have also been shown to cause an increase in the rate of adaptation (Hegreness et al., 2008). Currently, the vast majority of work done on this topic only addresses two drug combinations. This work's focus is on how three or more drug combinations can affect the evolution of antibiotic resistance.

Taking an evolutionary perspective to antibiotic resistance offers a unique understanding of how different combinatorial treatments affect the adaptation rates, frequencies, and resistance strengths of bacterial populations. The drug-drug interactions, along with the relative selection strength the combinations create, have an important effect on the evolution of antibiotic resistance (Oz et al., 2014; Tekin et al., 2017). With this information, knowing the rate resistance evolves and the process of resistance evolution itself, we can change treatment strategies to have resistance evolution work to our benefit. These strategies would lead to resistance to one treatment option in a bacterial population yet still remain susceptible to other antibiotic treatment options.

Antibiotic resistance is a good model for addressing fundamental questions in evolution. Microbial systems allow for detailed evolutionary experiments and the use of antibiotics allows for a variety of selection pressures to be used. With a system with so much versatility in factors and precision in data collection new insights into evolutionary biology can be made. This dissertation work offers an interdisciplinary approach to understanding of how higher-order interactions among antibiotics can affect the evolution of antibiotic resistance. While combinational drug therapy is still largely a phenomenological field, the use of quantitative approaches with an evolutionary perspective will continue to be instrumental in the growth of the field and progression towards resolving the increasing prevalence of multi-drug resistant bacteria. In addition to the biomedical relevance, this work is an attractive system to examine fundamental evolutionary questions dealing in adaptation dynamics. This is an especially enticing system with respect to the evolution of populations imposed by multiple simultaneous stressors (antibiotic combinations). The information gained from this dissertation can be used to

better inform predictions of evolutionary rates and trajectories which can be exploited in our effort to combat antibiotic-resistant pathogens.

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ORIGINAL ARTICLE

Evolutionary Applications Open Access WILEY

Evolution of antibiotic cross-resistance and collateral sensitivity in *Staphylococcus epidermidis* using the mutant prevention concentration and the mutant selection window

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Abstract

In bacteria, evolution of resistance to one antibiotic is frequently associated with increased resistance (cross-resistance) or increased susceptibility (collateral sensitivity) to other antibiotics. Cross-resistance and collateral sensitivity are typically evaluated at the minimum inhibitory concentration (MIC). However, these susceptibility changes are not well characterized with respect to the mutant prevention concentration (MPC), the antibiotic concentration that prevents a single-step mutation from occurring. We measured the MIC and the MPC for *Staphylococcus epidermidis* and 14 single-drug resistant strains against seven antibiotics. We found that the MIC and the MPC were positively correlated but that this correlation weakened if cross-resistance did not evolve. If any type of resistance did evolve, the range of concentrations between the MIC and the MPC tended to shift right and widen. Similar patterns of cross-resistance and collateral sensitivity were observed at the MIC and MPC levels, though more symmetry was observed at the MIC level. Whole-genome sequencing revealed mutations in both known-target and nontarget genes. Moving forward, examining both the MIC and the MPC may lead to better predictions of evolutionary trajectories in antibiotic-resistant bacteria.

KEYWORDS
antibiotic resistance, antimicrobial, bacterial evolution, correlated traits, susceptible

1 | INTRODUCTION

In recent decades, there has been a rapid increase in the prevalence of multi-antibioticresistant pathogens (Dijkshoorn, Nemec, Nemec, & Seifert, 2007; Leski et al., 2016; Nordmann, Naas, Naas, Fortineau, & Poirel, 2007; Tandogdu et al., 2016; Zalacain et al., 2016). This growing public health threat (Bush et al., 2011; Davies & Davies, 2010; Sanders, 2001; Woolhouse, Waugh, Waugh, Perry, & Nair, 2016) has made it necessary to better understand how evolution of resistance to one antibiotic affects bacterial

Lozano-Huntelman and Singh contributed equally.

susceptibility to other antibiotics (Pál, Papp, Papp, & Lázár, 2015). Increased resistance to one antibiotic frequently results in increased resistance to another antibiotic (Haight & Finland, 1952; Obolski, Stein, Stein, & Hadany, 2015; Sanders, 2001), termed cross-resistance. Conversely, increased resistance to one antibiotic can also often result in decreased resistance to another antibiotic (Obolski et al., 2015; Pál et al., 2015), a phenomenon referred to as collateral sensitivity. By understanding the factors that influence both types of collateral responses, we can better predict evolutionary trajectories of resistant mutants based on the antibiotics they have been exposed to.

There have been hundreds of previous studies on collateral responses, but the vast majority of them have examined these responses only in the context of minimum inhibitory concentration (MIC), which is the antibiotic concentration required to inhibit growth by a set amount (typically 99% inhibition; Barbosa, Beardmore, Beardmore, Schulenburg, & Jansen, 2018; Haight & Finland, 1952; Imamovic & Sommer, 2013; Obolski et al., 2015; Sanders, 2001; Sanders, Sanders, Sanders, Goering, & Werner, 1984; Thomson & Sanders, 1994). A small number of recent studies have started to also examine collateral effects at the mutant prevention concentration (MPC; Imamovic & Sommer, 2013; Podnecky et al., 2018), which is the concentration at which no single-step resistant mutant can occur (Baquero & Negri, 1997; Bush et al., 2011; Dong, Zhao, Zhao, Domagala, & Drlica, 1999; Drlica, 2003; Drlica & Zhao, 2007). This is often thought of as the concentration needed to prevent the evolution of antibiotic resistance in a typical population size infection of approximately 10^{10} cells (Dong, Zhao, Zhao, Kreiswirth, & Drlica, 2000).

For example, Imamovic and Sommer (2013) used gentamicin and cefuroxime to show that changes in MPC correlated with collateral responses in resistant mutants in *Escherichia coli*. A few years later, Podnecky et al. (2018) compared the MPC for 17 *E. coli* drug-strain combinations that showed conserved collateral responses. They found that in 12 of these cases, the change in MPC was consistent with the sign of the collateral responses. Moreover, the mutant selection window (MSW), which is the range of antibiotic concentrations that selects for single-step resistant mutants (Drlica, 2003; Drlica & Zhao, 2007) and that is bounded by the MIC at the lower end and the MPC at the upper end (Figure 1), was shown to shift up or down depending on the collateral response (Podnecky et al., 2018). Here, we examine networks of collateral responses at both the MIC level and the MPC level, focusing on whether collateral responses are symmetric or asymmetric and how these responses shift the MSW. To investigate these questions, we use 49 drug-strain combinations of *Staphylococcus epidermidis* (Winslow & Winslow, 1908).

Due to a scarcity of previous work examining the MPC as opposed to the MIC, there is a knowledge gap not only in our understanding of how collateral responses at the MIC and MPC levels differ but also in our understanding of correlated evolution between the MIC and MPC. A review of studies examining the correlation between the MIC and the MPC shows that there tends to be a low positive correlation between these traits (Drlica, Zhao, Zhao, Blondeau, & Hesje, 2006). However, the results have been shown to be species-dependent based on differing correlations in *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* (Drlica et al., 2006). If the MIC and the MPC are correlated in the data collected here, then selection pressure affecting the MIC could have indirect effects on the MPC for *S. epidermidis* (Brokordt, González, González, Farías, Winkler, & Lohrmann, 2017; Krebs, Feder, Feder, & Lee, 1998; Price & Langen, 1992). The correlations between the MIC and the MPC vary not only with the type of bacteria but also with the type of antibiotics used (Imamovic & Sommer, 2013; Podnecky et al., 2018).

Antibiotics can be categorized into classes based on their mechanisms of action (Chopra & Roberts, 2001; Davis, 1987; Gaynor & Mankin, 2003; Waxman & Strominger, 1983). Cross-resistance occurs within and across antibiotic classes (Haight & Finland, 1952; Obolski et al., 2015; Sanders, 2001; Thomson & Sanders, 1994). For example, cross-resistance within the quinolones occurs when the same cellular target has been altered (Martínez & Baquero, 2002; Ruiz, 2003; Sanders, 2001; Sanders et al., 1984). In the case of nalidixic acid-resistant bacteria, enhanced resistance to ciprofloxacin and norfloxacin is also displayed (Sanders et al., 1984). The resistant mutations to nalidixic acid are described as target modifiers and change the cellular target of the antibiotic to limit its effectiveness (Hemaiswarya, Kruthiventi, Kruthiventi, & Doble, 2008; Martínez & Baquero, 2002). Because of this, these types of mutations are considered effective against antibiotics with similar mechanisms of action (Martínez & Baquero, 2002; Ruiz, 2003; Sanders, 2001; Sanders et al., 1984).

When antibiotics have different mechanisms of action, resistance to one antibiotic does not necessarily cause resistance to another antibiotic. In quinolones, there are cases where resistance to one quinolone does not cause resistance to other quinolones. For example, ciprofloxacin's primary target in *S. pneumoniae* is topoisomerase IV and sparfloxacin's primary target is DNA gyrase. Single-step mutants selected by one of these antibiotics are less susceptible to the selecting antibiotic but not the other because of different mechanisms of resistance in response to different drug targets (Sanders, 2001).

While different mechanisms of action can sometimes reduce the likelihood of cross-resistance, this is not always the case. Cross-resistance across antibiotic classes can occur from mutations in genes that regulate efflux pumps, genes that change outer membrane proteins, or nontargeted mutations in a stress response pathway (Lázár et al., 2014, 2013; Obolski et al., 2015; Sanders et al., 1984). In one case, with quinolone-resistant *K. pneumoniae*, changes in the outer membrane proteins caused cross-resistance to beta-lactams (Sanders et al., 1984). Another study showed that fluoroquinolone-resistant *E. coli* containing mutations in a topoisomerase gene (*gyr*A) have changed susceptibility of the bacteria to other antibiotics. These changes include increases in resistance to ampicillin, cefoxitin, ciprofloxacin, nalidixic acid, kanamycin, and tobramycin and increases in sensitivity to nitrofurantoin and doxycycline (Lázár et al., 2014).

In addition to cross-resistance, bacteria can also exhibit collateral sensitivity to antibiotics (Lázár et al., 2013). Since collateral sensitivity

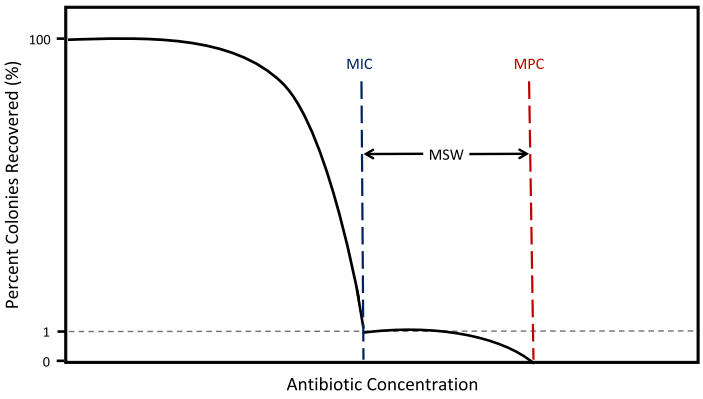

FIGURE 1 The mutant selection window (MSW) ranges from the MIC to the MPC. The MSW ranges from the MIC (blue line), inhibiting wild-type growth, to the MPC (red line), at which two simultaneous mutations are needed to survive. The MIC results in a 99% decrease in the numbers of recovered colonies, while MPC results in no recovered colonies. Selection for resistance mutations typically occurs within the MSW. Schematic adapted from Drlica and Zhao (2007)

TABLE 1 A list of antibiotics used and the median and range of the MIC (μg/ml) and MPC (μg/ml) values of the parental strain (*S. epidermidis* ATCC 14990)

Parental strain							
		MIC			MPC		
Antibiotics	Abbreviation	Median	Min	Max	Median	Min	Max
Ciprofloxacin	CPR	0.125	0.125	0.3	1	1	3.75
Doxycycline	DOX	2.6	2	3	16	12	16
Erythromycin	ERY	0.45	0.4	0.5	13	10	16
Gentamicin	GEN	0.293	0.234	0.351	9.36	9.36	9.36
Neomycin	NEO	1	1	1	17.5	15	20
Oxacillin	OX	0.12	0.105	0.12	0.6	0.6	0.6
Tetracycline	TET	8.75	6.25	15	125	125	125

occurs when resistance to one drug causes increased susceptibility to other drugs, it is considered an evolutionary trade-off (Bollenbach, 2015; Pál et al., 2015). For example, cellular uptake of aminoglycosides relies on the proton motive force (PMF). As a result, a reduction in the PMF is frequently the mechanism underlying resistance to aminoglycosides. However, efflux pumps responsible for removing other antibiotics also rely on the PMF. Therefore, resistance to aminoglycosides (due to a reduction in the PMF) can increase susceptibility to other antibiotics, typically expelled through efflux pumps (Pál et al., 2015). In recent years, new resistome studies have demonstrated that the pool of resistance genes is extraordinarily large (Dantas & Sommer, 2014). Characterizing the genomes of the antibiotic-resistant bacteria examined here is thus important to uncovering new mechanisms of cross-resistance and collateral sensitivity.

In this study, we ask four main questions. First, is there a correlation between the MICs and MPCs? Second, when resistance to a single antibiotic evolves, how does the MSW change? Third, how do cross-resistance and collateral sensitivity networks at the MIC level compare to these networks at the MPC level? Is symmetry (i.e., when a strain is resistant to drug A and cross-resistant to drug B, a strain that is resistant to drug B is also cross-resistant to drug A) more prevalent at one level than the other? Finally, what are the mutations that are associated with cross-resistance and collateral sensitivity? To answer these questions for *S. epidermidis*, we used seven antibiotics that covered five different mechanisms of action (Table 1). We spontaneously evolved two resistant mutants per antibiotic, resulting in 14 spontaneous mutant-resistant strains of *S. epidermidis*. For each of the 14 strains, we determined the MIC, MPC, and thus, the MSW for all seven antibiotics. We then sequenced their genomes and identified mutations affecting resistance.

2 | MATERIALS AND METHODS

2.1 | Bacteria and antibiotics

We collected spontaneous mutants by evolving *S. epidermidis* (ATCC 14990) to each of the seven antibiotics listed in Table 1 separately. *S. epidermidis* was plated on 150-mm agar plates with antibiotic concentration ranging from 2 × liquid MIC and ending at 20 × liquid MIC in increments of 2 × liquid MIC estimate. Colonies were selected off the highest concentration where colonies were recovered, in experiments where there was a clear and definable MPC with no colonies recovered after an achieved concentration. We then streak-purified the colonies from the spontaneous mutant experiments onto separate plates containing antibiotic

concentrations above the known MIC to confirm resistance. We inoculated a single colony into LB media (10 g tryptone, 5 g yeast extract, and 10 g NaCl). We then stored this culture in 25% glycerol at −80°C (Mayfield et al., 2013; Taylor & Webster, 2009). We initiated all experiments from a freshly thawed aliquot of this single batch.

We obtained and purified two independent spontaneously resistant mutants for each antibiotic, resulting in 14 resistant strains. The resistant strains were named based off of the antibiotic used to select for them. For example, the two strains resistant to ciprofloxacin were labeled as CPR R1 and CPR R2. We termed these "spontaneous mutant-resistant strains."

We further independently evolved *S. epidermidis* (ATCC 14990) to each of the seven antibiotics in Table 1. We evolved eight strains to each antibiotic for about 100 generations, resulting in 56 adapted resistant strains. We evolved the strains in a step-wise manner where the antibiotic concentration was continually doubled from ½ × MIC to 8 × MIC every 48 hr over the course of 10 days. We termed these "adapted resistant strains." This was done to capture the possibility of mutation acquisition being dependent on the dose of antibiotic the bacteria were exposed to (Jahn, Munck, Munck, Ellabaan, & Sommer, 2017; Lindsey, Gallie, Gallie, Taylor, & Kerr, 2013; Oz et al., 2014).

2.2 | Liquid MIC

We obtained MICs for the parental *S. epidermidis* ATCC 14990 strain and all 70 resistant strains (spontaneous and adapted) for every antibiotic assessed in this study. We created a liquid culture using 2 ml of LB in a culture tube and adding 150 µl of the thawed cell culture aliquot. We then placed this tube in a shaker set at 220 revolutions per minute (RPM) and 37°C to incubate until the OD_{600} reached 0.3 (Tecan Infinite M200 PRO Multimode Microplate Reader). We loaded fresh LB media and the selected antibiotic at varying concentrations into a 96-well plate to have a volume of 100 µl per well. We diluted bacterial cultures by a factor of 1:500 to create the inoculum. We added 100 µl of the inoculum to each well resulting in a final volume of 200 µl per well. We measured bacterial growth by reading the OD after 18 hr and defined the MIC as the minimum antibiotic concentration observed to inhibit growth by at least 95% among all replicate wells. We included both positive (LB + bacteria) and negative (LB only) controls on each plate to ensure bacterial growth of the particular strain and no contamination of media. We used these measurements to obtain a rough estimate of the MIC to determine MIC in agar, as described below.

2.3 | Agar MIC and MPC assays

2.3.1 | Bacterial preparation

We prepared the cultures from a single freezer aliquot (250 µl) by inoculating into 10 ml of LB. We grew the cultures overnight for 18 hr at 37°C and 160 RPMs. Afterward, we inoculated the entire bacterial culture into 450 ml of fresh LB until an OD600 between 0.45 and 0.70 was reached. Then at 4°C, we centrifuged the cultures at about 3,000 *g* for 10 min to obtain a high concentration of cells when plating and set aside the supernatant. We re-suspended the pellet in 7.5 ml of the original supernatant (Figure S1A).

2.3.2 | Determining agar MIC

Because there may be discrepancies between the liquid MIC estimate and agar MIC, we measured MIC in agar simultaneously with MPC experiments. Since identical increments were taken in each biological replicate, little variation would arise due to the liquid MIC estimate. The liquid MIC and agar MIC only differed slightly (±0.5 µg/ml) when increments of at least twofold were used. We prepared agar plates using 1,000 ml of autoclaved Milli-Q water with 15 g agar powder and one 25 g LB tablet (10 g tryptone, 5 g yeast extract, 10 g NaCl, and 1.5 g/L Tris/Tris-HCl).

To determine MIC, we plated 100-mm petri plates with 20 ml of LB agar with antibiotics ranging from 0.2 × liquid MIC estimate to 1.7 × liquid MIC estimate in increments of 0.1 × liquid MIC estimate (Figure S1B). We inoculated each of these plates with 10^5 CFU via sterile glass beads following the Copacabana method (Mills, Gareau, & Garcia, 2005; Worthington, Luo, & Pelo, 2001) and included a positive control containing no antibiotic. We incubated the plates at 37°C for 72 hr, and colonies were counted. We used two replicates, and following another study (Tan et al., 2009), we defined the MIC in agar as the first antibiotic concentration where the number of colonies was reduced by 95% or greater from the control in both of the two plates. While many studies use the 99% cutoff (Haight & Finland, 1952; Obolski et al., 2015; Sanders, 2001; Thomson & Sanders, 1994), we used a slightly lower cutoff to account for random noise in the data. For each drug-strain combination, we determined the MIC in three separate instances resulting in six plates. We recorded the median and range for each MIC.

2.3.3 | MPC determination and analysis

To determine the MPC, we used three 150-mm plates with 60 ml of LB agar for each antibiotic concentration ranging from 2 × liquid MIC estimate and ending at 20 × liquid MIC estimate in increments of 2 × liquid MIC estimate (Figure S1C). We then inoculated the plates with 10^{10} CFUs via sterile glass beads following the Copacabana method (Worthington et al., 2001). We defined the MPC as the lowest antibiotic concentration where there was no growth across all three replicates (Allen, Kaatz, Kaatz, & Rybak, 2004; Dong et al., 1999; Drlica, 2003; Drlica & Zhao, 2007; Firsov, Lubenko, Lubenko, Smirnova, Strukova, & Zinner, 2008; Firsov et al., 2003; Hansen, Zhao, Zhao, Drlica, & Blondeau, 2006; Metzler et al., 2004). We conducted the MPC assays in triplicates resulting in a total of nine agar plates per drug-strain combination. We calculated both the median and the range.

2.4 | Mutant selection window

With the MIC and MPC values determined, we measured the MSW in terms of the parental MIC value. This allowed us to directly compare how the MSW changes across multiple strains.

2.5 | Whole-genome sequencing

We performed whole-genome sequencing on the parental strain of *S. epidermidis* ATCC 14990 and on all spontaneous mutant-resistant strains. The sequences were paired-end with a length of 150 bp. We aligned the sequences to the *S. epidermidis* ATCC 12228-reference genome to elucidate the genetic changes underlying their antibiotic-susceptibility phenotypes. We used *S. epidermidis* ATCC 12228 as the reference genome due to its more complete gene annotation. We streak-purified all strains on LB agar plates prior to DNA library preparation and *HiSeq* sequencing at the Genewiz Next Generation Sequencing facility in South Plainfield, New Jersey. We note that most of the plasmids in the reference genome, *S. epidermidis* ATCC 12228, are not represented in the *S. epidermidis* ATCC 14990 strains. However, the smallest plasmid, NC_005008 (4,439 bp), is fully represented as a circular element in all strains and carries a tetracycline resistance gene and two replication protein genes (Putonti et al., 2017). Sequencing coverage shows most strains have five copies of this plasmid. However, DOX R1, DOX R2, and TET R2 appear to have 12–16 copies (Tables S2 and S3). One of the parental strains (parental strain 2) appears to have lost the plasmid and has one tenth of the main chromosome coverage. We suspect this may be due to the plasmid being lost during cultivation for sequencing for parental strain 2.

2.6 | Bioinformatics analysis

We removed the adapter sequences from sequence reads, and the quality was checked using Trim Galore! (http://www.bioinformatics.babraham.ac.uk) with quality trimming turned off. Trim Galore! is a wrapper for cutAdapt (Martin, 2011) and FastQC (https://www.bioinformatics.babraham.ac.uk). We mapped trimmed reads using BWA-MEM v.0.7.12-r1039 (Li & Durbin, 2010) to the *S. epidermidis* ATCC 12228 genome (2,499,279 bp chromosome & 6 plasmids, 4,439–24,365 bp, NCBI Accessions NC_004461.1 and NC_005003-8). All samples had at least 97% of the adapter trimmed reads mapped to the ATCC 12,228 genome. We performed variant discovery and filtering with GATK v 3.7-0-gcfedb67 (McKenna et al., 2010), including MarkDuplicates, HaplotypeCaller in GVCF mode with ploidy 1, GenotypeGVCFs, and finally VariantFiltration with the following hard filters applied: QD < 20.0, MQ < 40.0, FS > 60.0, SOR > 3.0, MQRankSum < −12.0, ReadPosRankSum < −8.0. SnpEff (Cingolani et al., 2012) was used to determine the context of the variants and predict the functional impact. We removed variants with an allele frequency of 1 across all of the strains including the two parent strains with GATK's SelectVariants. We used the VCFtools package (Danecek et al., 2011) to inspect summaries of the filter's effects and the transition transversion ratios for each.

After manual inspection of alignments, we excluded additional variants from regions highly divergent from the reference genome, as the alignments in these regions are unreliable mainly due to structural rearrangements. These excluded regions are main chromosome positions 37885-38551, 57541-57702, 91802-93606, 200225, 666092, 1519681-1519683, 2311095-2312854, and 2471276-2471507. We used GATK's DepthOfCoverage to determine mean depth of coverage across each sample and across each genomic element (Tables S2 and S3).

3 | RESULTS

3.1 | Correlated evolution of the MIC and MPC

We found an increase in the median MIC and the median MPC (compared with the parental strain) for all 14 spontaneous mutant-resistant strains of *S. epidermidis* (Table 1) except TET R2. For both the MIC and the MPC for this strain, we were unable to determine values due to an extremely high level of resistance. Kendall's rank correlations of the MIC and the MPC data were used to evaluate any possible relationship between the MIC and MPC due to the data heteroscedasticity. The overall correlation of the MICs and MPCs showed that as the MIC increased, the MPC increased ($\tau = .5510332$, $p < 2.2 \times 10^{-16}$; Figure 2). This trend holds true when examining each individual spontaneous mutant-resistant strain across all antibiotics using Kendall's rank correlation ($p < .05$ for each strain), with the exception of doxycycline and tetracycline (Figure S2).

We observed that the outcomes of evolution affected this correlation. If resistance evolved, through direct selection or through cross-resistance, the correlation remained roughly the same as the overall correlation between all MICs and MPCs ($\tau = .5238549$, $p < 7.3 \times 10^{-9}$; Figure 3a). However, if no cross-resistance evolved, observed through no change in the MIC or through instances of collateral sensitivity, the correlation between MIC and MPC became weaker ($\tau = .3438369$, $p < .025$; Figure 3b).

The mixture of bactericidal and bacteriostatic antibiotics used could have confounded the relationship between MIC and MPC. Bactericidal drugs are ciprofloxacin, oxacillin, and gentamicin, and bacteriostatic drugs are doxycycline, erythromycin, and tetracycline. We found no difference in the size of the MSW and no difference in the fold change in MIC or MPC between bactericidal and bacteriostatic drugs. Neomycin has both bactericidal and bacteriostatic activities so we left it out of our analysis.

3.2 | Changes in the mutant selection window

We compared the MSWs using the median MIC and the median MPC for the parental and spontaneous mutant-resistant strains across all antibiotics (Figure 4). When resistance evolved, regardless of

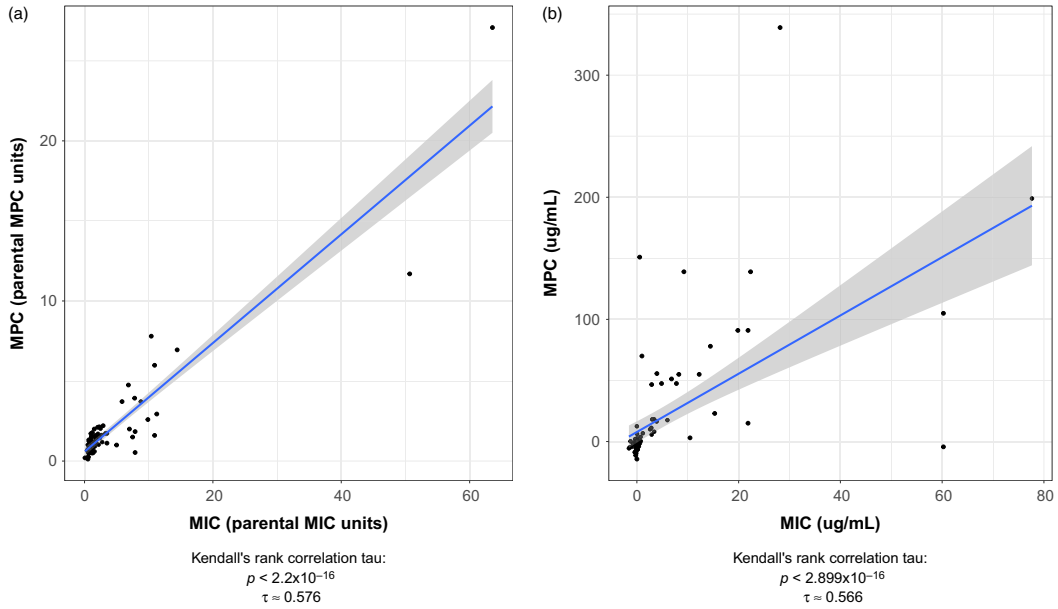

FIGURE 2 A positive correlation is found between MIC and MPC in *Staphylococcus epidermidis*. The MIC is plotted against the MPC in (a) parental MIC and parental MPC units (e.g., $MIC_{strain}/MIC_{parent}$ and $MPC_{strain}/MPC_{parent}$) and (b) μg/ml. A positive correlation was found (Kendall rank correlation test, [a] $\tau = .576$, $p < .001$, [b] $\tau = .566$, $p < .001$)

whether it was through direct resistance to drug X or through cross-resistance, the MSW shifted right and widened. Paired *t* tests were used to evaluate both the increase in the MIC ($p < .0005$) indicating the right shift and the increase in range of the MSW ($p < .05$) indicating the widening of the MSW. This was a general trend of the MSW and is seen when resistance is selected for or when cross-resistance evolves either within an antibiotic class (i.e., gentamicin/neomycin and tetracycline/doxycycline) or across classes (i.e., DOX R1 and TET R1 exposed to oxacillin). However, when there is no evolved cross-resistance or when there are cases of collateral sensitivity at the MIC, the MSW does not follow the trend of shifting right and widening. In these cases, the MSW either narrows or behaves in a highly variable way.

For example, when treated with erythromycin, only the ERY R1 and ERY R2 strains had a larger MIC and wider MSW. All other spontaneous mutant-resistant strains treated with erythromycin appeared to have MSWs that narrowed or were unchanged when compared to the parental strain (Figure 4g). We showed that collateral sensitivity to erythromycin at the MPC level frequently occurred, while the MIC was essentially not affected (Figure 4g). This means that the MSW for erythromycin narrowed for most of the spontaneous mutant-resistant strains other than erythromycin-resistant ones.

Another exception to the pattern of the MSW widening and shifting right appeared for strains treated with oxacillin (Figure 4d). Of these strains, only OX R1 and OX R2 consistently showed resistance and a widening of the MSW. The MSW of all other spontaneous mutant-resistant strains treated with oxacillin seems to vary in size dramatically and has no consistent trend.

3.3 | MPC cross-resistance and collateral sensitivity

To investigate instances of cross-resistance and collateral sensitivity, we used the MPC ranges to create a network map of the types of cross-resistance (Figure 5, Table 1 and Table S1). We define cross-resistance as a rightward shift in the range of the spontaneous mutant-resistant strains, where these strains and the parental strain ranges do not overlap ($max_{parent} < min_{resistant\ strain}$). Collateral sensitivity is a downward shift in the range of the spontaneous mutant-resistant strains, where these strains and the parental strain ranges do not overlap ($max_{resistant\ strain} < min_{parent}$).

Cross-resistance was observed a total of 25 times and at least once in each spontaneous mutant-resistant strain (Figure 5a). Cross-resistance was found in both of the spontaneous mutant-resistant strains (R1 and R2) 64% of the time for the same antibiotic. We found cross-resistance to antibiotics within and across different classes (Figure 5a). Patterns of cross-resistance among antibiotics of the same class have already been observed at the MIC level (Sanders et al., 1984), and most of these patterns are preserved when considering the MPC values (Figure 5).

Collateral sensitivity was found in both spontaneous mutant-resistant strains (R1 and R2) 62% of the time. Regarding collateral

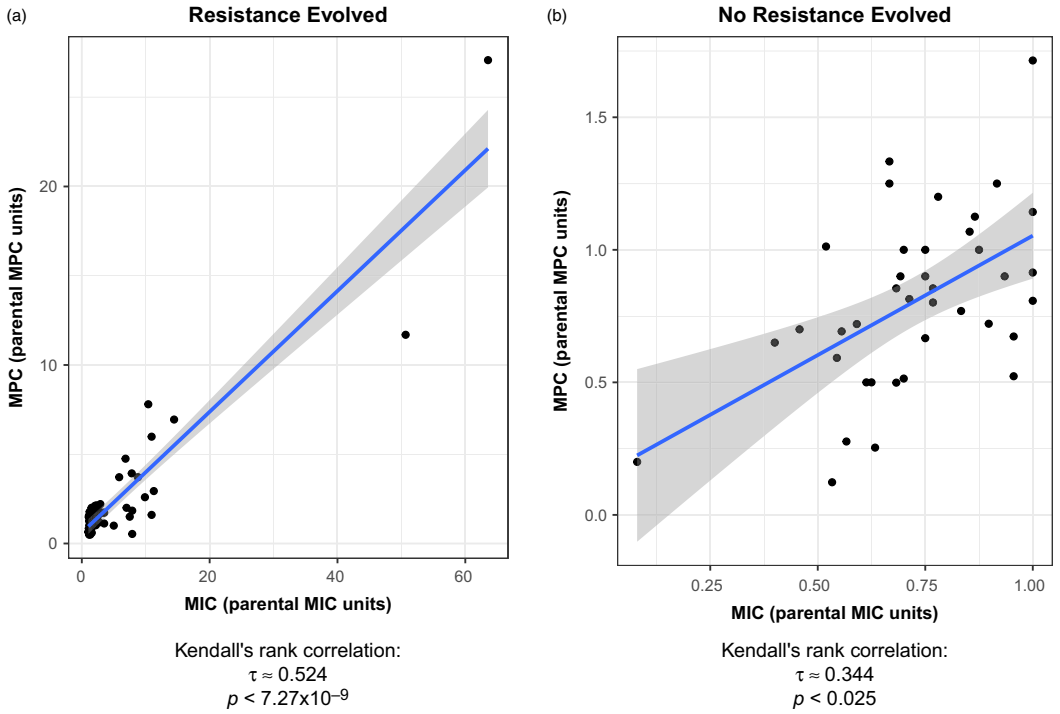

FIGURE 3 The positive correlation found between MIC and MPC weakens if cross-resistance has not evolved. The fold change after evolution of the MIC medians ($MIC_{strain}/MIC_{parent}$) is plotted against the fold change after evolution of the MPC medians ($MPC_{strain}/MPC_{parent}$) for (a) spontaneous mutant-resistant strains that showed evolved resistance at the MIC (MIC fold change >1; Kendall rank correlation test, $\tau = .524$, $p < .001$) and (b) spontaneous mutant-resistant strains that did not show evolved cross-resistance at the MIC (MIC fold change ≤1). A positive correlation between the change in the MIC and the change in the MPC was found in both instances, but the correlation was weaker when cross-resistance did not evolve (Kendall rank correlation test, $\tau = .344$, $p < .025$)

sensitivity, our main findings were as follows: (a) collateral sensitivity to erythromycin and gentamicin was common (Figure 5b), and (b) resistance to doxycycline was generally associated with collateral sensitivity to nontetracycline antibiotics (neomycin, gentamicin, oxacillin, and erythromycin; Figure 5b).

The adapted resistant strains showed extremely high cross-resistance to all antibiotics, and the MICs for these adapted resistant strains were so high that they exceeded the maximum solubility for some of the antibiotics used (Please see the supplemental information for more detailed methods and results; Appendix S1 and Figure S1).

Next, we asked whether there were any cases of symmetrical cross-resistance and/or symmetrical collateral sensitivity and if the resulting networks were similar at the MIC and MPC levels. A symmetrical relationship is defined as having the same type of cross-resistance for each set of resistant strains to their complimentary antibiotic. For example, a symmetrical relationship would occur if a strain is resistant to antibiotic A and cross-resistant to antibiotic B, and a different strain is resistant to antibiotic B and cross-resistant to antibiotic A. Cases of symmetrical cross-resistance and collateral sensitivity can be viewed as a positive feedback loop. Symmetrical cross-resistance positively reinforces resistance to either antibiotic, whereas symmetrical collateral sensitivity positively reinforces susceptibility to either antibiotic.

We found that symmetrical relationships were more prevalent at the MIC level (five cross-resistant and five collaterally sensitive symmetries) than at the MPC level (three of each symmetry type; Figure 5). We identified two possible symmetrical relationships of cross-resistance within the same antibiotic classes of tetracyclines (tetracycline and doxycycline) and aminoglycosides (neomycin and gentamicin), both of which were observed at the MIC and MPC level (Figure 5a,c). We also identified two possible symmetrical relationships between classes: an MPC cross-resistance symmetry between the tetracyclines (tetracycline and doxycycline) and the beta-lactam (oxacillin) and an MPC collateral sensitivity symmetry between the aminoglycosides (neomycin and gentamicin) and the beta-lactam (oxacillin). Both of these symmetrical relationships between classes were only constantly observed at the MPC level (Figure 5a,b).

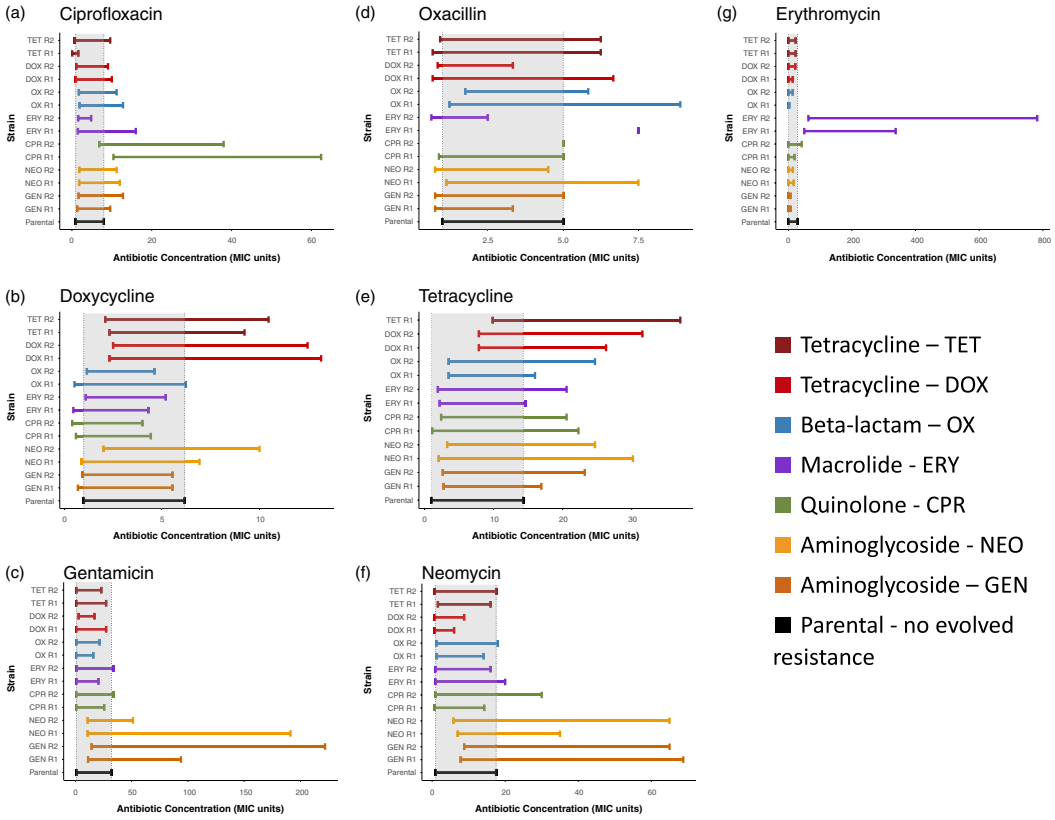

FIGURE 4 The MSW tends to shift to the right and widen as resistance evolves. The gray regions indicate the mutant selection windows of the parental strain. The MSW for each spontaneous mutant-resistant strain is shown in panels (a–g), which are divided by the antibiotic used to determine the MSW. As resistance evolves, the MSW tends to shift to the right and widen as compared to the parental strain (gray-shaded region). When cross-resistance does not evolve, the MSW is highly variable. In Panel (d), ERY R1 and CPR R2 have MSWs that appear as single points because the median MIC and median MPC for these strains are the same, so the MSW has a size of zero. Given the large antibiotic concentration increments used in this study, it is very likely that the true values lie in between the increments. In Panel (e), the TET R2 MSW is missing because the MIC and MPC for tetracycline of the TET R2 were undetermined due to high levels of resistance

3.4 | Mutations in the genome

We found thirteen unique antibiotic resistance mutations. Nine were missense mutations, and the remaining four consisted of disruptive in-frame insertions, mutations in the upstream region, changes in plasmid copy number, or stop codons (Table 2). Resistance typically occurs through mutations within a target gene. The spontaneous mutant-resistant strains CPR R1, CPR R2, ERY R1, ERY R2, GEN R1, GEN R2, NEO R1, and NEO R2 all gained mutations in genes that are associated with resistance to their respective antibiotic (Besier, Ludwig, Ludwig, Brade, & Wichelhaus, 2003; Bodley, Zieve, Zieve, Lin, & Zieve, 1969; Chittum & Champney, 1995; Davydova, Streltsov, Streltsov, Wilce, Liljas, & Garber, 2002; Sreedharan, Peterson, Peterson, & Fisher, 1991). We found instances of resistance that may be due to novel or nontarget mutations (SE_p103, SE0706, SE608, SE1860, SE2021) and are shared between both strains (Table 2).

4 | DISCUSSION

4.1 | Effects of resistance on MIC and MPC

Previous research has yielded much information about collateral responses measured using MICs (Haight & Finland, 1952; Imamovic & Sommer, 2013; Obolski et al., 2015; Sanders, 2001; Sanders et al., 1984; Thomson & Sanders, 1994). Here, we examined whether and how the MIC and the MPC are related, how the MSW changes as resistance evolves, and what the patterns of collateral responses at the MPC level are.

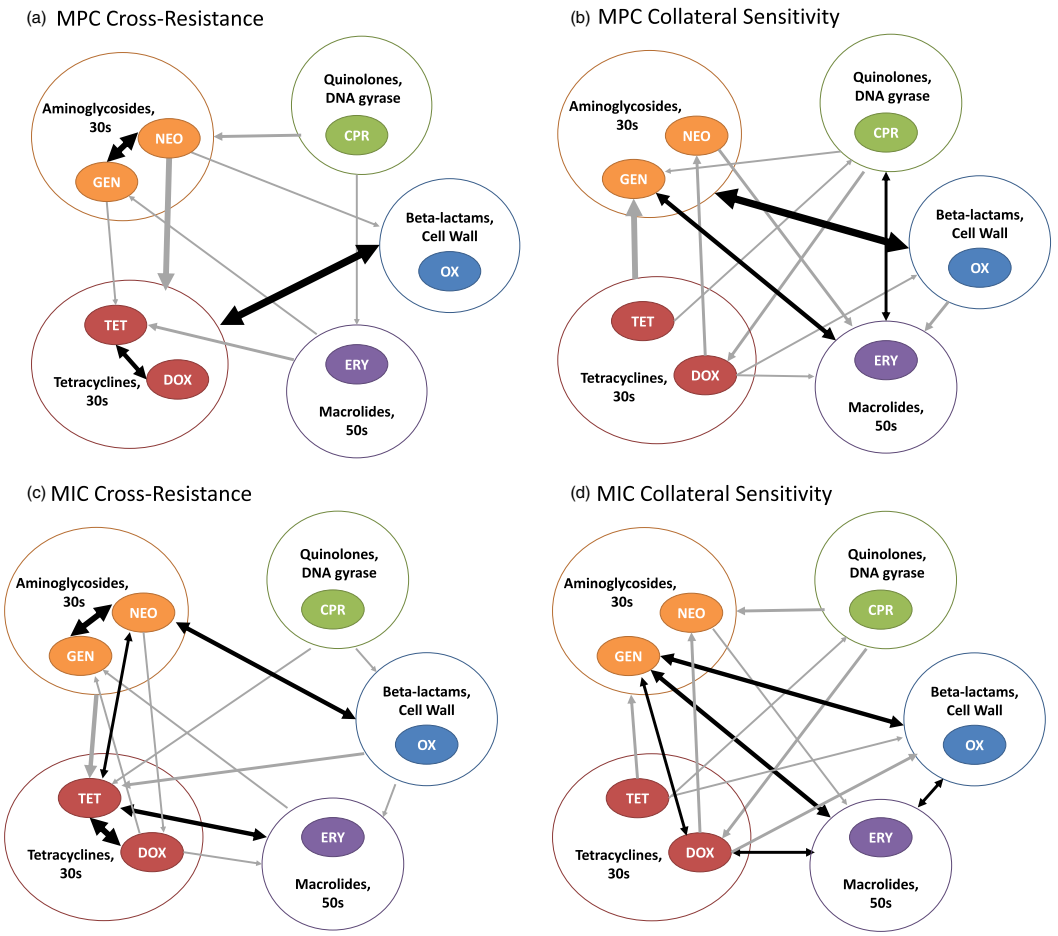

FIGURE 5 Symmetrical relationships are more prevalent at the MIC level than at the MPC level. This figure design is based on Pál et al. (2015) showing the network maps of the types of MPC and MIC cross-resistance and collateral sensitivity. Arrows represent the presence, amount, and direction of the outcomes. Arrows originate at the selective antibiotic of a resistant strain and end at the antibiotic susceptibility being tested. Black double arrows highlight symmetrical relationships. Arrows may originate and end at the larger circles encompassing one to two antibiotics; this indicates all respective strains or antibiotics exhibit the same relationships. The weight of each arrow indicates the number of outcomes exhibiting the same relationship. (a) MPC cross-resistance patterns. (b) MPC collateral sensitivity patterns. (c) MIC cross-resistance patterns. (d) MIC collateral sensitivity patterns. Both cross-resistance and collateral sensitivity were identified both within and across antibiotic classes. The collateral response networks show similar patterns at the MIC and MPC levels, but the MIC level has notably more symmetry (five symmetrical cross-resistances and five symmetrical collateral sensitivities) than the MPC level (three symmetrical cross-resistances and three symmetrical collateral sensitivities)

The widespread correlation between the MIC and the MPC (Figure 2) in the spontaneous resistant strains suggested that as selection acts on the MIC, indirect selection occurs at the MPC level in *S. epidermidis*. This is consistent with previous work correlating these concentrations in other bacterial species (Drlica et al., 2006). Intriguingly, our results suggest that evolution of resistance affects that correlation. We find that the overall positive correlation of the MIC and MPC is strongly held when resistance is evolved (τ = .5238549, $p < 7.3 \times 10^{-9}$) but becomes substantially weaker when cross-resistance has not evolved (τ = .3438369, p < .025; Figure 3).

That is, if the collateral result of resistance evolution does not increase the MIC, the correlation weakens. Since the overall correlation is relatively strong as MIC increases, we expect and observe that the MPC increases as well. But if the MIC decreases, there is a much lower likelihood that the MPC will decrease as well. Although

TABLE 2 Resistance conferring mutations

Resistance	Associated gene or plasmid	Strains	Type of mutation	References	Chromosomal position	Nucleotide substitution	Amino acid substitution
Ciprofloxacin	*SE1037*	CPR R1	Missense	Sreedharan et al. (1991)	1045114	C → A	p.Ser80Tyr
		CPR R2		Ferrero, Cameron, and Crouzet (1995)		C → T	p.Ser80Tyr
Erythromycin	*rplV*	ERY R1 ERY R2	Disruptive in-frame insertion	Chittum and Champney (1995) Davydova et al. (2002)	1863928	G → GCTTGTACGTTTATTAATTGCA	p.Ser101_His102insAlaIleAsnLysArgThrSer
Aminoglycoside	*fusA*	GEN R1	Missense	Bodley et al. (1969)	310912	G → T	p.Arg59Leu
		GEN R2	Missense		312466	C → A	p.Ser577Tyr
		NEO R1 NEO R2	Missense	Besier et al. (2003)	312253	A → C	p.Tyr506Ser
Tetracycline	*NC_005008*	TET R2	Change in plasmid copy number	Putonti et al. (2017)	n/a	n/a	n/a
	SE0075	TET R1 TET R2	Stop gained	*	74230	C → T	p.Gln281*
	SE2021	TET R1 TET R2	Missense	*	2059908	G → C	p.Met201Ile
Doxycycline	*SE_p103*	DOX R1 DOX R2	Upstream gene variant	*	3147	T → C	n/a
	SE0706	DOX R1 DOX R2	Missense	*	696607	G → A	p.Arg70Lys
	SE608	DOX R1 DOX R2	Missense	*	595362	G → T	p.Ala85Ser
	SE1860	DOX R1 DOX R2	Missense	*	1898875	G → A	p.Ala238Val
Oxacillin	*SE2021*	OX R1 OX R2	Missense	*	2059908	G → C	p.Met201Ile

*Mutations that have not yet been identified and/or confirmed in the literature.

there is still a significant correlation in the cases where the collateral result of resistance evolution does not increase the MIC, this positive correlation is seen only about 30% of the time (Figure 3b). It is important to note that the correlation between MIC and MPC (using all cases where resistance evolved and where it did not evolve) is not significant for tetracycline and doxycycline (Figure S3), underscoring the importance of testing this correlation between each antibiotic–bacteria combination.

Our observed pattern of the MSW generally shifting has also been observed in *E. coli* (Podnecky et al., 2018). However, it has not previously been reported in the context of collateral responses that the MSW shifts and widens. This pattern may be important for understanding the effects of aggressive treatment strategies like using high drug dosages (Read, Day, Day, & Huijben, 2011). Reducing bacterial load through these strategies can make it easier for a patient's immune system to defeat an infection and decrease the probability of de novo mutations that confer resistance from arising (Drlica, 2003; Read et al., 2011). However, if highly resistant mutants already exist within the original infection or if de novo mutants arise that are highly resistant, aggressive antibiotic treatment applies the strongest possible selection for these mutants. This gives highly resistant mutants the best possible chance of repopulating the infection and spreading to other people (Drlica, 2003; Read et al., 2011). Our finding that the MSW shifts right and widens as resistance evolves provides important context for this work. It suggests that when high concentrations of an antibiotic are used, the range of concentrations that selects for resistant mutants generally increases and makes the resulting mutants even more resistant (Drlica, 2003).

Oz and colleagues further demonstrated the implications of high antibiotic concentrations on resistance using isogenic *E. coli* populations. In their study, they evolved two populations under strong selection and two populations under mild selection for each of 22 antibiotics over 3 weeks. Upon constructing cross-resistance networks, they found that bacterial populations that had evolved antibiotic resistance under strong selection demonstrated higher levels of cross-resistance than those that had evolved antibiotic resistance under milder selection (Oz et al., 2014). Our result is consistent with their finding: Mutants selected at the MPC level generally displayed MSWs that widened and shifted to the right when exposed to other antibiotics. Taken together, these findings suggest that combination drugs are likely to be more effective than ever-increasing dosages of a single drug when considering the role that selective pressure can have on collateral effects (Oz et al., 2014) and the size of the resulting MSWs (Michel, Yeh, Chait, Moellering, & Kishony, 2008).

4.2 | Cross-resistance and collateral sensitivity at the network level

We found that there are more symmetrical relationships at the MIC level than at the MPC level. The MPC symmetries tended to be a subset of the MIC symmetries. This may be because spontaneous mutant-resistant strains were originally selected at the MIC level, and although MIC and MPC are positively correlated, the MPC did not always increase with the MIC. In cases where cross-resistance did not evolve, or where there was collateral sensitivity, the MPC did not increase along with the MIC and the symmetrical relationships were not preserved at the MPC level. Additionally, the correlation between MIC and MPC was not perfect and varied depending on the antibiotic (Figure S2), so this also contributed to MIC symmetrical relationships not always carrying over to the MPC level.

Our finding of symmetrical MPC cross-resistance within tetracyclines and the aminoglycosides (Figure 5a) and MPC cross-resistance between different antibiotic classes is congruent with previous work conducted using MICs (Pál et al., 2015). For example, it has been shown that *E. coli* K12 strains resistant to tetracycline or chloramphenicol exhibited a decreased sensitivity to fluoroquinolones (Cohen, McMurry, McMurry, Hooper, Wolfson, & Levy, 1989), and our findings at the MPC level support this.

Our results at the MPC level for collateral sensitivity (Figure 5b) also support results from a previous study that used the MIC values to find cases of collateral sensitivity across antibiotics with various mechanisms of action in *E. coli* (Lázár et al., 2014). Our findings make sense when viewed in light of studies showing that collateral responses are relatively stable as resistance develops (Munck, Gumpert, Gumpert, Wallin, Wang, & Sommer, 2014). Recent work suggests that collateral sensitivity and cross-resistance may be even more important than drug interactions when it comes to using drug combinations to combat resistance (Munck et al., 2014; Rodriguez de Evgrafov, Gumpert, Gumpert, Munck, Thomsen, & Sommer, 2015). This is because drug interaction types change as resistance develops but the mechanisms behind collateral responses are more stable (Munck et al., 2014; Rodriguez de Evgrafov et al., 2015).

For example, a study examining six antibiotics and five antibiotic pair combinations found no relationship between drug interaction type and resistance evolution beyond wild-type levels of resistance, but found that cross-resistance and collateral sensitivity were important in predicting resistance evolution (Rodriguez de Evgrafov et al., 2015). Upon examining the genomes of *E. coli* that were evolved in the presence of five different antibiotics and the resulting 10 antibiotic pairs, it was found that collaterally sensitive drug combinations consistently created environments in which mutants resistant to either antibiotic were counterselected, and thus, there was decreased evolution of resistance overall (Munck et al., 2014).

4.3 | Genes involved in resistance

We found that some spontaneous mutant-resistant strains had mutations within the same genes, yet show distinct phenotypic variation. For example, TET and OX spontaneous mutant-resistant strains conferred an identical mutation on *SE2021*, an amino acid transporter gene (Zhang et al., 2003), yet have phenotypic differences in the MSW in the presence of doxycycline (Table 2 and Figure 4). The MSW of TET shifts to the right and widens compared

with the MSW of OX, which remains the same as the wild-type MSW (Figure 4b).

Additionally, DOX R1 and DOX R2 were genetically identical, but we observed a case where DOX R1 was exposed to oxacillin and the strain showed MPC collateral sensitivity against one drug (oxacillin), while DOX R2 showed MPC collateral sensitivity against a different drug (erythromycin). Differing responses of cross-resistance and collateral sensitivity among replicates have been observed in other experiments (Barbosa et al., 2017), and whole-genome sequencing revealed distinct evolutionary paths of resistance in these cases (Barbosa et al., 2017). Since the liquid MIC for oxacillin was determined to be 0.08 μg/ml, the low MIC value may have affected the accuracy of measuring the MPC in this case. The MPC, unlike the MIC, is not a single value but could vary significantly due to Luria-Delbruck fluctuations (Gianvecchio et al., 2019; Jones, Thomas, Thomas, & Rogers, 1994; Luria & Delbrück, 1943).

Despite this variation in MPC values, we generally found that patterns of the types of cross-resistance are common within antibiotic classes at both the MIC and MPC levels, which may be attributed to the types of mutations they share. For example, both aminoglycoside resistance strains, GEN and NEO (R1 and R2), had different mutations on the same gene *fusA*, a ribosomal gene originally identified as conferring resistance to fusidic acid (Table 2; O'Neill, Larsen, Larsen, Henriksen, & Chopra, 2004). GEN and NEO spontaneous mutant-resistant strains showed similar phenotypic responses across the seven drugs even though the individual mutations resulted in an amino acid change in different locations within *fusA*. Studies have shown that there are many different mutations within *fusA* that result in resistance to fusidic acid and have similar MICs (Laurberg et al., 2000), yet the specific amino acid substitutions that we have identified here have not previously been reported. However, *fusA* has also been reported as encoding for an elongation factor that is responsible for increased resistance in both *E. coli* (Zengel, Archer, Archer, & Lindahl, 1984) and *T. thermophilus* (Laurberg et al., 2000). We suspect that this characteristic could also play a role in the resistance phenotypes of the GEN and NEO spontaneous mutant-resistant strains of *S. epidermidis*. Although we did not look into other traits, such as fitness costs, associated with these genomic changes, we believe that future work can help explain the observed phenotypic variation. Further genomic characterizations can help identify more genetic mechanisms underlying cross-resistance and collateral sensitivity (Hickman, Munck, Munck, & Sommer, 2017).

4.4 | Potential clinical applications

Since the MSW typically broadens under antibiotic treatment, this suggests that typical treatment strategies, which use antibiotic concentrations well above the MIC based on the antibiotic's pharmacokinetic and pharmacodynamics values (Bonhoeffer, Lipsitch, Lipsitch, & Levin, 1997; Levison & Levison, 2009), can potentially select for mutations that confer greater resistance. This indicates the limited utility of using ever-increasing dosages of a single drug to narrow the MSW.

Notably, we found that when there was no evolved cross-resistance or when there were cases of collateral sensitivity at the MIC, the MSW did not follow the trend of shifting right and widening. In the case of oxacillin-treated strains, only OX R1 and OX R2 consistently showed resistance and a widening of the MSW. All other strains had MSWs that did not follow a consistent trend (Figure 4d). When examining this figure, it is important to note that ERY R1 and CPR R2 have MSWs that appear as single points because their median MIC and median MPC were the same, resulting in MSWs of size zero. Variation within Figure 4d highlights the importance of testing each antibiotic to understand its effect on resistant strains rather than assuming that all antibiotics will cause the MSW to shift and widen.

Another interesting case of the MSW not following this general trend occurred with the erythromycin-treated strains. Here, the MSW narrowed or stayed nearly the same for all spontaneous mutant-resistant strains except erythromycin-resistant ones (Figure 4g). Even though resistance to erythromycin can become extremely strong, it may be a good option for the treatment of infections that are already resistant to another antibiotic. For these infections, there would be a reduced chance of subsequently evolving cross-resistance to erythromycin, as evidenced by the narrowed range of concentrations in which further single-step resistant mutants could evolve in our experiments. It is interesting to note that the MSW of CPR R2 widened slightly in response to erythromycin rather than narrowing like the MSW of other strains (Figure 4g). The deviation from this general trend may be due to the difference in the point mutation in SE1037 within the CPR spontaneous mutant-resistant strains (Table 2). Antibiotics that tend to gain collateral sensitivity in the MPC and to shrink the MSW, such as erythromycin, may be a good component for an antibiotic cycling therapy or combinational therapy.

Collateral sensitivity and cross-resistance are frequently observed not only in the laboratory but also in clinical settings. For example, a study examining resistance in 2,478 *E. coli* isolates from urinary tract infections found high levels of cross-resistance between many pairs of drugs, including gentamicin and ampicillin, ciprofloxacin and sulfamethoxazole, and trimethoprim and sulfamethoxazole (Kahlmeter & Menday, 2003). Separate work that also examined resistance in *E. coli* isolates from urinary tract infections used 16 antibiotics and observed 141 instances of cross-resistance (e.g., between ciprofloxacin and chloramphenicol and between nitrofurantoin and amoxicillin) and 92 instances of collateral sensitivity (e.g., between ciprofloxacin and gentamicin and between ciprofloxacin and colistin; Podnecky et al., 2018).

Clinicians can potentially take advantage of collateral sensitivity through antibiotic cycling or combination therapy. Cycling between antibiotics that demonstrate collateral sensitivity may prevent the fixation of mutations that result in stronger resistance to one antibiotic, and may also result in hypersensitivity to other

antibiotics (Imamovic & Sommer, 2013). Our findings of potential symmetrical collaterally sensitive relationships suggest two-drug sets of antibiotics to use in further investigations of the antibiotic cycling strategy, including oxacillin and gentamicin. For example, oxacillin may initially be effective at killing a bacterial population, but with repeated exposure, resistance to this drug will likely evolve. If the bacterial population is then treated with gentamicin and evolves resistance to this new drug, it may become susceptible to oxacillin again. This type of antibiotic cycling strategy, that is, taking advantage of collateral sensitivity, may help extend the usefulness of currently available antibiotics (Bush et al., 2011; Davies & Davies, 2010; Gonzales et al., 2015; Imamovic & Sommer, 2013; Sanders, 2001).

However, when considering a cyclic approach to treating bacterial infections, it is also important to take into consideration our finding that the MPC does not correlate as strongly to the MIC, and thus, the MSW does not behave in a predictable way when cross-resistance does not evolve for spontaneous mutant-resistant strains. Since cyclic treatment strategies depend on resistance to new drugs not evolving due to collateral sensitivity (Imamovic & Sommer, 2013), the MPC should be evaluated for each step of the cycle. This could help ensure that dosage concentrations are not within the new MSW to account for cases in which the MSW widens even if the MIC decreases.

Our results can expand on the cycling strategy by identifying potential cases of symmetrical collateral sensitivity using the MSW across seven antibiotics that span five classes. Symmetrical cases of collateral sensitivity can be much more useful than asymmetrical ones, because the order in which a population of bacteria evolves resistance matters less, since there is collateral sensitivity in both directions (Imamovic & Sommer, 2013). Due to the small number of replicates we use here and evidence that collateral sensitivity patterns in laboratory strains do not always apply to clinical isolates (Imamovic & Sommer, 2013), it is important to conduct further studies using clinical isolates. Furthermore, bacteria are not typically selected at MPC concentrations in clinical settings because the toxicity resulting from such high concentrations is too much for the human body to handle (Blondeau, Zhao, Zhao, Hansen, & Drlica, 2001; Gianvecchio et al., 2019; Metzler et al., 2004).

In conclusion, we have shown how the mutant prevention concentration (MPC) and the mutant selection window (MSW) change for a range of drugs after the evolution of resistance to one antibiotic in *S. epidermidis*. When examining our data for each spontaneous mutant-resistant strain, we found that the MSWs tend to shift right and widen as antibiotic resistance evolves, showing a strong correlation between the MIC and MPC. However, the MSW varies dramatically and the correlation between the MIC and MPC weakens when cross-resistance has not evolved at the MIC. When examining our data at the network level, we found that cross-resistance and collateral sensitivity patterns within MIC and MPC networks are similar, and there are more cases of symmetrical relationships at the MIC level than at the MPC level. Our genetic analysis of the strains used here further supports the importance of traditional target-gene mutations and reveals possible novel or nontarget mutations in antibiotic resistance evolution. Overall, using both the MIC and the MPC to evaluate antibiotic resistance may lead to better predictions of the evolutionary outcomes of resistant mutants when exposed to different antibiotics.

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CONFLICT OF INTEREST

None declared.

DATA AVAILABILITY STATEMENT

The phenotypic assay data for this study are available in the supporting information, and the whole-genome sequencing data are available on NCBI's SRA (Accession Number: PRJNA593298).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Supplemental Information

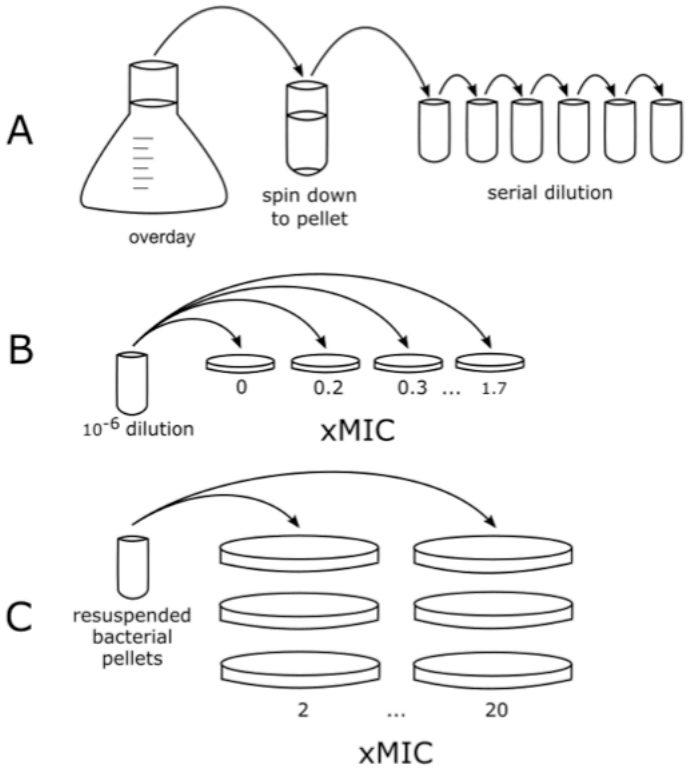

Figure 2-6. *Supplemental Figure 1.* Overview of the MPC assay procedure. A) The cell culture is grown, centrifuged down, re-suspended, and diluted to the desired concentration. B) MIC is determined through inoculation of a 10-6 dilution on 100 mm plates. The lowest concentration inhibiting at least 95% of growth relative to the control (no antibiotic) plate is considered to be the MIC. **C)** MPC is determined through inoculation of approximately 10^{10} CFUs on larger 150mm plates. The MPC value is considered to be the lowest concentration resulting in no growth.

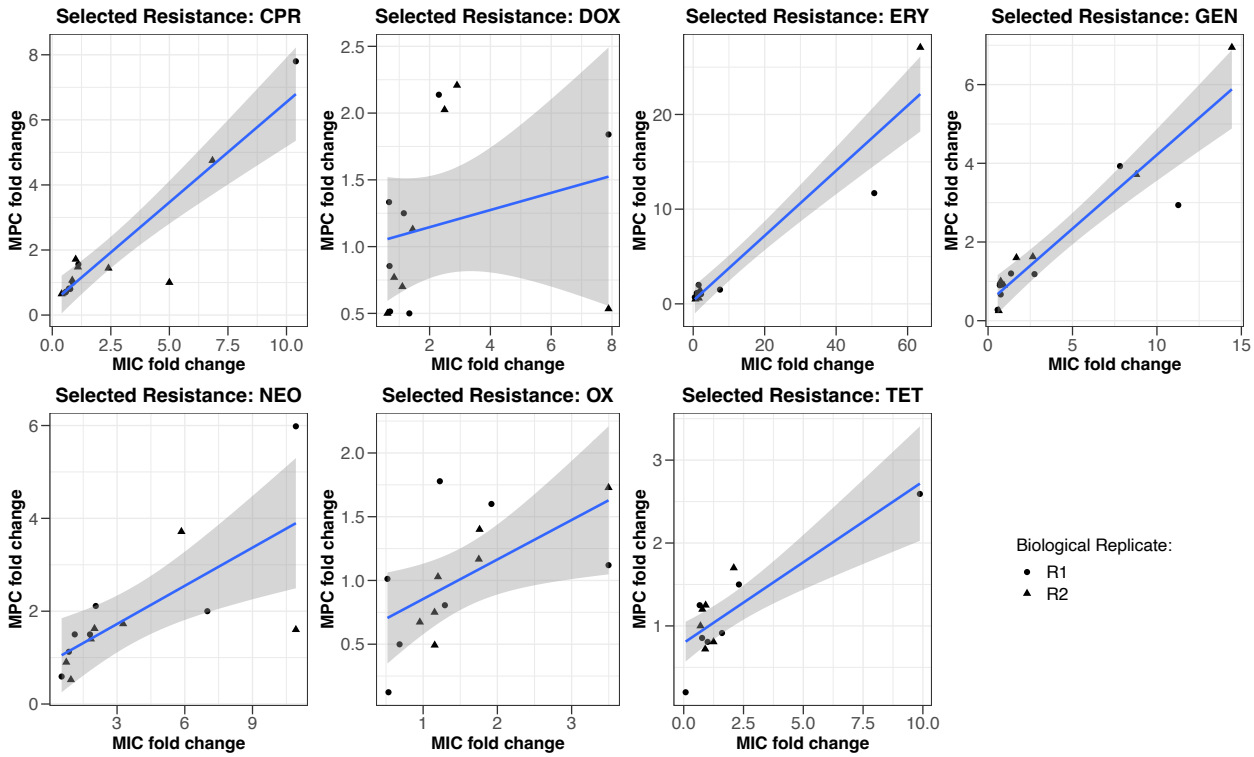

Figure 2-7. *Supplemental Figure 2.* Correlation of the MIC and MPC Tends to Be High Across Selected Resistance Groups.The fold change after the evolution of the MIC medians ($MIC_{strain}/MIC_{parent}$) is plotted against the fold change after evolution in the MPC medians ($MPC_{strain}/MPC_{parent}$). The shapes represent which strain the median was determined for; circles are R1 and triangles are R2. A Kendall's rank correlation test was performed for each set of spontaneous mutant resistant strains: CPR ($\tau = 0.729, p < 2\ x\ 10^{-4}$), DOX ($\tau = 0.322, p > 0.05$), ERY ($\tau = 0.552, p < 0.01$), GEN ($\tau = 0.789, p < 9.678\ x\ 10^{-5}$), NEO ($\tau = 0.7, p < 0.001$), OX ($\tau = 0.575, p < 0.005$), TET ($\tau = 0.39, p > 0.05$). Even though the correlations between MIC and MPC are significant overall, they vary by antibiotic. For the tetracyclines (DOX and TET), the correlations are not significant.

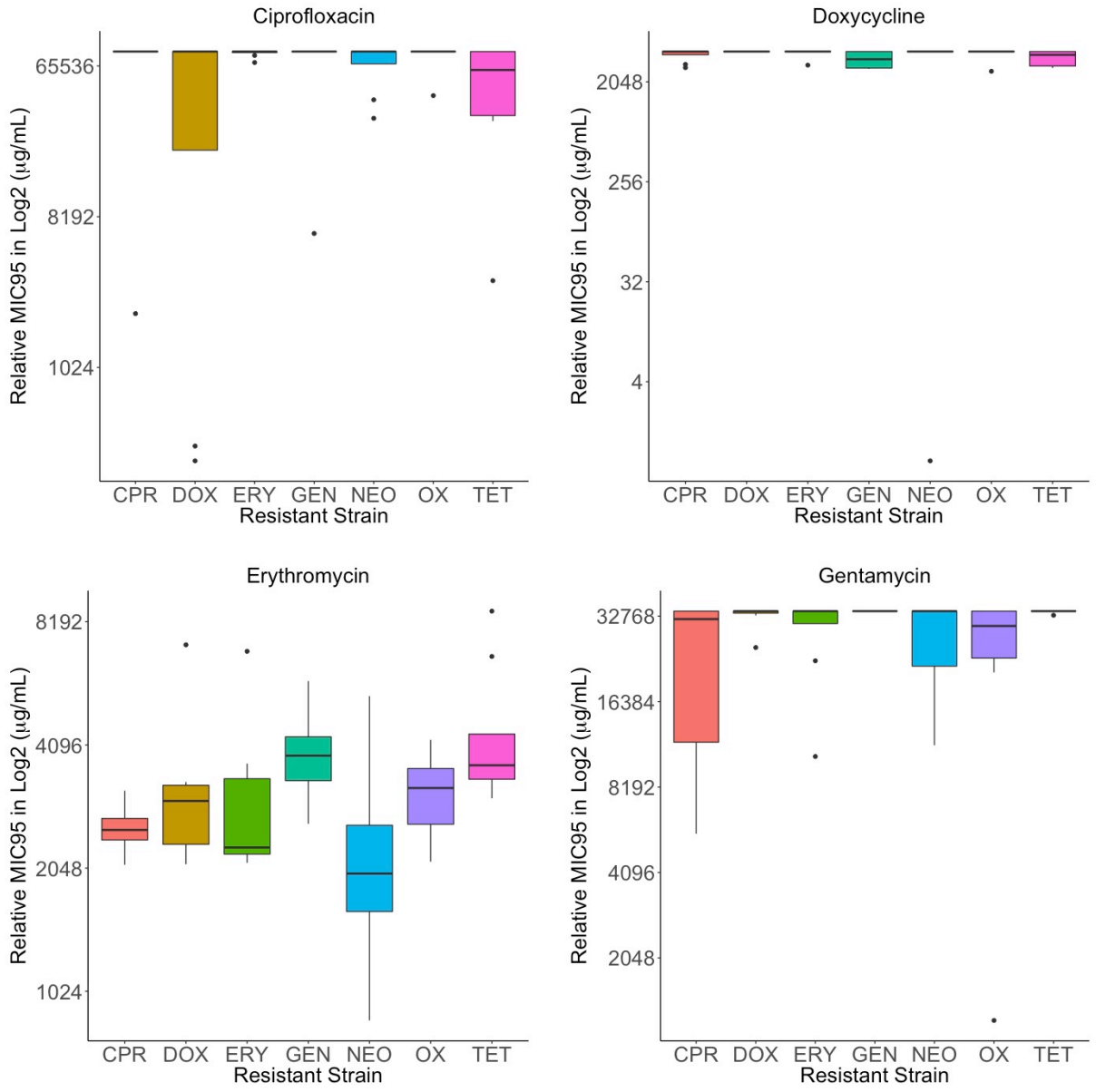

Figure 2-8. *Supplemental Figure 3.* Adapted Resistant Strains Show High Levels of Cross-Resistance to All Antibiotics. Relative MIC_{95} values of adapted resistant strains are shown to be very high. Resistant strains are presented in the x-axis, grouped according to antibiotic of selected resistance. Data for the eight biological replicates were grouped by the antibiotic of selected resistance. The relative MIC µg/mL (observed MIC/ancestral MIC) is presented on the y-axis in a Log2 scale. Any MIC value that exceeded the threshold of 10,000µg/mL was

assigned the value of 10,000μg/mL. MIC values for the adapted resistant strains were close to or above solubility thresholds, making MPC experiments with these strains not feasible.

Table 2-3. *Supplemental Table 1.* Median and ranges of MIC (μg/mL) and MPC (μg/mL) of all spontaneous mutant resistant strains. Bolded text indicates instances of resistance. Red bold indicates cross-resistance, determined by non-overlapping ranges. Blue italicized indicates collateral sensitivity, as determined by non-overlapping ranges when compared to parental strains.

Spontaneous Mutant Resistant Strains																	
strain	Antibiotic	MIC			MPC			strain	Antibiotic	MIC			MPC		
		median	min	max	median	min	max			median	min	max	median	min	max
CPR R1	**CRP**	**1.3**	**1.04**	**1.3**	**7.8**	**7.28**	**7.8**	CPR R2	**CRP**	**0.855**	**0.76**	**1.045**	**4.75**	**3.42**	**4.75**
	DOX	*1.536*	*1.152*	*1.92*	*11.52*	*7.68*	*11.52*		*DOX*	*1.04*	*0.78*	*1.04*	*10.4*	*10.4*	*10.4*
	ERY	0.25	0.15	0.4	*9*	*9*	*9*		ERY	0.493	0.384	0.548	**19.18**	**19.18**	**21.92**
	GEN	*0.225*	*0.225*	*0.225*	*7.5*	*7.5*	*7.5*		GEN	0.25	0.2	0.3	**10**	**10**	**10**
	NEO	*0.713*	*0.713*	*0.95*	14.25	9.5	23.75		NEO	1	1	1	**30**	**25**	**30**
	OX	0.105	0.105	0.12	0.6	0.6	0.6		**OX**	**0.6**	**0.6**	**0.9**	0.6	0.6	0.9
	TET	9.75	7.8	11.7	195	117	195		**TET**	**21**	**18**	**27**	180	120	240
ERY R1	CRP	0.19	0.175	0.225	2	2	2.5	ERY R2	CRP	0.195	0.195	0.195	*0.6*	*0.6*	*0.6*
	DOX	*1.19*	*0.98*	*1.4*	11.2	11.2	14		DOX	2.85	2.85	2.85	13.5	12	15
	ERY	**22.8**	**18.24**	**25.84**	**152**	**121.6**	**152**		**ERY**	**28.6**	**28.6**	**28.6**	**352**	**172**	**352**
	GEN	0.3	0.3	0.3	*6*	*6*	*7.5*		**GEN**	**0.4**	**0.4**	**0.4**	**10**	**10**	**10**
	NEO	1	0.75	1	20	20	20		NEO	1	1	1	16	16	16
	OX	0.9	0.075	0.9	0.9	0.6	1.5		*OX*	*0.075*	*0.075*	*0.075*	0.3	0.3	0.6
	TET	**19.2**	**16**	**22.4**	**128**	**128**	**192**		**TET**	**17**	**17**	**17**	**180**	**180**	**180**
NEO R1	CRP	0.225	0.225	0.225	1.5	1.5	1.5	NEO R2	CRP	0.23	0.21	0.23	1.4	1.4	1.75
	DOX	2.25	2.25	2.25	**18**	**18**	**18**		**DOX**	**5.2**	**5.2**	**5.2**	**26**	**26**	**26**
	ERY	*0.245*	*0.245*	*0.245*	*7.7*	*7.7*	*7.7*		ERY	0.43	0.34	0.51	*6.8*	*6.8*	*8.5*
	GEN	**3.2**	**3.2**	**3.2**	**56**	**56**	**56**		**GEN**	**3.2**	**3.2**	**3.2**	**15**	**15**	**15**
	NEO	**7**	**7**	**7**	**35**	**35**	**35**		**NEO**	**5.85**	**3.25**	**5.85**	**65**	**52**	**91**
	OX	**0.135**	**0.135**	**0.135**	**0.9**	**0.9**	**0.9**		OX	0.09	0.07	1	*0.54*	*0.54*	*0.54*
	TET	**18**	**18**	**18**	**264**	**264**	**264**		**TET**	**28.6**	**23.4**	**28.6**	**216**	**216**	**216**
TET R1	*CRP*	*0.01*	*0.01*	*0.01*	*0.2*	*0.15*	*0.25*	TET R2	CRP	0.0975	0.075	0.12	1.2	1.2	1.2
	DOX	**6**	**4.8**	**7.2**	**24**	**34**	**36**		**DOX**	**5.44**	**5.44**	**7.48**	**27.2**	**27.2**	**27.2**
	ERY	0.45	0.45	0.6	10.5	10.5	13.5		**ERY**	**0.56**	**0.525**	**0.6**	10.5	9	12
	GEN	*0.225*	*0.2*	*0.225*	*8*	*7*	*8*		GEN	0.263	0.225	0.3	*6.75*	*4.5*	*9*
	NEO	**1.6**	**1.6**	**1.8**	16	12	24		*NEO*	*0.7*	*0.525*	*0.875*	17.5	10.5	17.5
	OX	*0.08*	*0.08*	*0.08*	**0.75**	**0.75**	**1**		OX	0.11	0.075	0.1375	0.75	0.5	1.25
	TET	**86.4**	**86.4**	**86.4**	**324**	**324**	**324**		**TET**	**liq**	**liq**	**liq**	**>200**	**200**	**200**
DOX R1	CRP	0.125	0.125	0.163	1.25	1.25	1.25	DOX R2	CRP	0.138	0.125	0.15	1.13	1	1.25
	DOX	**5.99**	**5.13**	**6.84**	**34.2**	**34.2**	**34.2**		**DOX**	**6.48**	**5.94**	**7.02**	**32.4**	**32.4**	**32.4**
	ERY	**0.6**	**0.6**	**0.6**	*6.5*	*6*	*7*		ERY	0.65	0.50	0.8	10	10.00	14
	GEN	*0.2*	*0.2*	*0.2*	*8*	*8*	*8*		**GEN**	**0.85**	**0.70**	**1**	*5*	*4.00*	*6*
	NEO	*0.7*	*0.6*	*0.9*	*6*	*6*	*12*		*NEO*	*0.613*	*0.525*	*0.7*	*8.75*	*7*	*10.5*
	OX	*0.08*	*0.07*	*0.09*	**0.8**	**0.8**	**0.8**		*OX*	*0.1*	*0.1*	*0.1*	*0.4*	*0.4*	*0.4*
	TET	**69**	**69**	**69**	**230**	**230**	**230**		**TET**	**69**	**69**	**69**	**276**	**276**	**276**
OX R1	CRP	0.24	0.24	0.24	1.6	1.2	2	OX R2	CRP	0.22	0.22	0.22	1.4	1.2	1.6
	DOX	*1.35*	*1.35*	*1.35*	**16.2**	**16.2**	**18.9**		DOX	3	3	3	12	12	12
	ERY	*0.24*	*0.24*	*0.24*	*1.6*	*1.6*	*1.6*		**ERY**	**0.52**	**0.52**	**0.52**	*6.4*	*4.8*	*6.4*
	GEN	*0.2*	*1-0*	*0.2*	*4.67*	*4-5*	*5*		GEN	0.28	0.28	0.28	*6.3*	*6.3*	*6.3*
	NEO	**1.293**	**1.175**	**1.41**	*14.1*	*9.4*	*14.1*		**NEO**	**1.2**	**1.2**	**1.2**	18	18	24
	OX	**0.147**	**0.12**	**0.16**	**1.067**	**0.8**	**1.6**		**OX**	**0.21**	**0.21**	**0.21**	**0.7**	**0.7**	**0.7**
	TET	**30.6**	**30.6**	**30.6**	**140**	**140**	**180**		**TET**	**30.6**	**30.6**	**30.6**	**216**	**216**	**252**
GEN R1	CRP	0.17	0.16	0.18	1.2	1.2	1.2	GEN R2	CRP	0.21	0.2	0.22	1.6	1.6	1.6
	DOX	*1.8*	*1.8*	*1.8*	14.4	14.4	14.4		DOX	2.43	2.16	2.7	14.4	14.4	14.4
	ERY	*0.255*	*0.24*	*0.27*	*3.6*	*3.6*	*3.6*		*ERY*	*0.285*	*0.27*	*0.3*	*3.3*	*2.4*	*4.2*
	GEN	**3.3**	**3.3**	**3.3**	**27.5**	**22**	**33**		**GEN**	**4.23**	**3.9**	**4.55**	**65**	**65**	**65**
	NEO	**7.813**	**6.25**	**9.375**	**68.75**	**50**	**87.5**		**NEO**	**8.775**	**7.8**	**9.75**	**65**	**65**	**78**
	OX	*0.09*	*0.08*	*0.1*	*0.4*	*0.4*	*0.4*		*OX*	*0.09*	*0.08*	*0.1*	**0.6**	**0.6**	**0.6**
	TET	**24.05**	**20.35**	**27.75**	148	111	185		**TET**	**23.2**	**21.75**	**24.65**	**203**	**203**	**203**

Table 2-4. *Supplemental Table 2.* Mean coverage across the main chromosome and plasmids.

Target	Total Coverage	Average Coverage	CPR R1	CPR R2	DOX R1	DOX R2	TET R1	TET R2	NEO R1	NEO R2	GEN R1	GEN R2	ERY R1	ERY R2	OX R1	OX R2	parental 1	parental 2
NC_004461.1:1-2499279	60403517981	24168.38	1427.71	1440.08	1578.9	1467.91	1441.01	1580.67	308.87	1493.61	1723.12	1338.39	1801.43	387.47	1729.66	340.33	1711.43	1536.14
NC_005008.1:1-4439	674233319	151888.56	7511.31	6159.05	20524.63	18334.83	6490.4	25178.53	1251.98	6380.82	8391.02	7027	8893.61	2221.88	9847.82	1811.94	7127.48	77.05
NC_005007.1:1-4679	287200	61.38	3.66	4.71	3.86	3.37	4.37	4.72	0.52	3.89	2.96	3.78	4.24	1.06	4.13	0.75	4.84	3.17
NC_005003.1:1-6585	115736	17.58	1.16	0.97	0.94	0.9	1.03	1.64	0.02	1.75	0.93	1.25	1.13	0	1.23	0.01	1.31	0.92
NC_005006.1:1-8007	116	0.01	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NC_005005.1:1-17261	193088089	11186.38	651.68	901.27	617.12	638.02	856.7	831.13	145.82	553.68	525.59	650.1	722.73	214.9	905.28	163.74	745.01	613.13
NC_005004.1:1-24365	53498254	2195.7	133.92	158.2	122.24	128.23	166.23	147.32	25.04	123.86	146.15	134.11	154.88	33.39	151.82	28.65	144.13	119.19

Table 2-5. *Supplemental Table 3.* Plasmid coverage is divided by the main chromosome coverage.

Target	CPR R1	CPR R2	DOX R1	DOX R2	TET R1	TET R2	NEO R1	NEO R2	GEN R1	GEN R2	ERY R1	ERY R2	OX R1	OX R2	parental 1	parental 2
NC_005008.1:1-4439	5.3	4.3	13.0	12.5	4.5	15.9	4.1	4.3	4.9	5.3	4.9	5.7	5.7	5.3	4.2	0.1
NC_005007.1:1-4679	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
NC_005003.1:1-6585	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
NC_005006.1:1-8007	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
NC_005005.1:1-17261	0.5	0.6	0.4	0.4	0.6	0.5	0.5	0.4	0.3	0.5	0.4	0.6	0.5	0.5	0.4	0.4
NC_005004.1:1-24365	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Supplemental Appendix 1. Liquid MIC Estimates for Independently Evolved Strains.

We obtained estimates of the MICs for the 56 independently evolved resistant strains for every antibiotic assessed in this study. We created a liquid culture using 2mL of LB in a culture tube and added 150µL of the thawed cell culture aliquot. We then placed this tube in a shaker set at 220 revolutions per minute (RPM) and 37°C to incubate until the OD_{600} reached 0.3 (Tecan Infinite M200 PRO Multimode Microplate Reader). We loaded fresh LB media and the selected antibiotic at varying concentrations into a deep-well 96-well plate to have a volume of 200µL per well. We diluted bacterial cultures by a factor of 1:500 to create the inoculum. We added 200µL of the inoculum to each well resulting in a final volume of 400µL per well. After 18hrs we aspirated, transferred 200ml of each well into a 96-well plate, and measured bacterial growth by reading the OD. We defined the MIC as the minimum antibiotic concentration observed to inhibit growth by at least 95% amongst all replicate wells. We included both positive (LB + bacteria) and negative (LB only) controls on each plate to ensure bacterial growth of the parental strain and no contamination of media. We used these measurements to obtain a rough estimate of the MIC to determine MIC in agar, as described in the methods.

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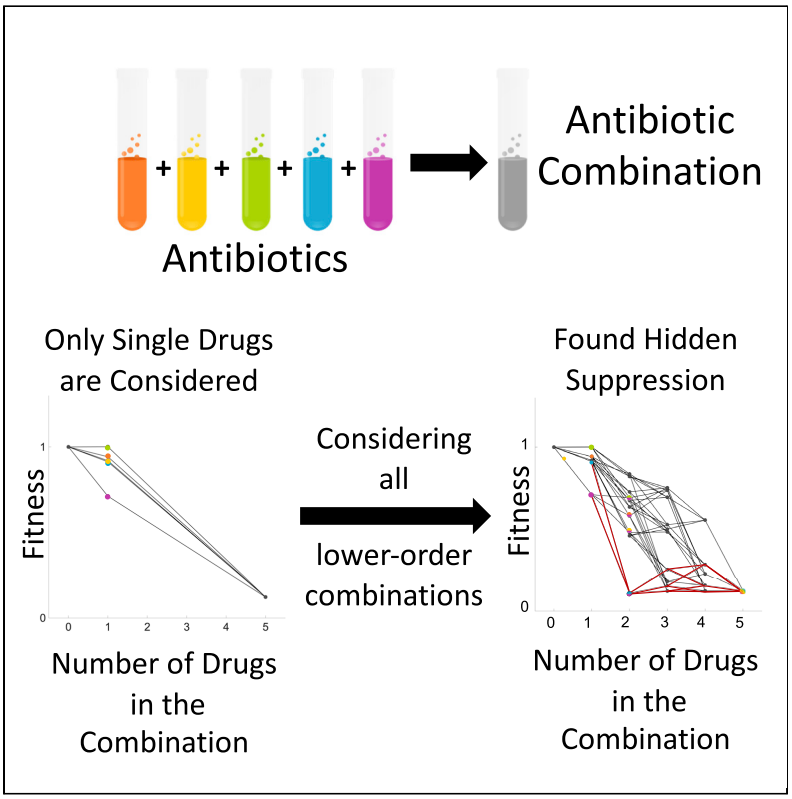

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Highlights

Most instances of suppressive interactions are missed by standard methods

A majority (54%) of all antibiotic combinations tested contain hidden suppression

Identifying hidden suppression can affect what combinations should be used

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Hidden suppressive interactions are common in higher-order drug combinations

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SUMMARY

The rapid increase of multi-drug resistant bacteria has led to a greater emphasis on multi-drug combination treatments. However, some combinations can be suppressive—that is, bacteria grow faster in some drug combinations than when treated with a single drug. Typically, when studying interactions, the overall effect of the combination is only compared with the single-drug effects. However, doing so could miss "hidden" cases of suppression, which occur when the highest order is suppressive compared with a lower-order combination but not to a single drug. We examined an extensive dataset of 5-drug combinations and all lower-order—single, 2-, 3-, and 4-drug—combinations. We found that a majority of all combinations—54%—contain hidden suppression. Examining hidden interactions is critical to understanding the architecture of higher-order interactions and can substantially affect our understanding and predictions of the evolution of antibiotic resistance under multi-drug treatments.

INTRODUCTION

As the numbers of multi-drug resistant bacteria continue to increase globally (Bloom et al., 2018; Chokshi et al., 2019; Povolo and Ackermann, 2019), there has been a greater emphasis on multi-drug treatments (Fischbach, 2011; Rieg et al., 2018; Liu et al., 2020). Two or more drugs interact in three main ways: additively, synergistically, or antagonistically. Additive combinations are when no interaction between drugs occurs; the combined effect is as expected, assuming each drug is acting independently (Bliss, 1939). A synergistic interaction occurs when two drugs work better than expected based on each single drug's effects, resulting in decreased bacterial fitness. An antagonistic interaction occurs when two drugs are less effective at killing bacteria in combination than expected based on each single drug's effects (Box 1).

The most extreme form of antagonism, termed suppression, occurs when bacterial growth increases with a combination of stressors rather than one single stressor alone (Yeh et al., 2006; Chait et al., 2007). This phenomenon was first described over a century ago when Fraser (1870) showed that two different toxins if administered by themselves would normally kill a rabbit but combined would keep the rabbit alive. Fraser termed this "physiological antidote" to indicate that this was not the result of a chemical interaction of the two toxins but rather an interaction of the two chemicals' effects on the physiology of the organism (Fraser, 1870, 1872a, 1872b).

However, for over a century, the idea of one drug being an antidote for another was mostly ignored. More recently, in the last decade and a half, there has been a renewed interest in this phenomenon of suppression (Yeh et al., 2006; Chait et al., 2007; Cokol et al., 2014; de Vos and Bollenbach, 2014; Bollenbach, 2015; Singh and Yeh, 2017; Lukačišin and Bollenbach, 2019; Tyers and Wright, 2019; Dean et al., 2020). Suppression was first defined in terms of antibiotic interactions when a systematic study of 2-drug interactions in 21 antibiotics was conducted, and approximately 10% of all interactions fell into the category of suppression (Yeh et al., 2006). Since then, there has been significant new work published on suppressive interactions and their effects on the evolution of resistance, their mechanisms, and their prevalence.

Multiple advances have been made in the conceptual and experimental tools used to identify drug interactions that have yielded intriguing results about suppressive interactions (Yeh et al., 2006; Toprak et al., 2013; Cokol et al., 2014; Tekin et al., 2016; Katzir et al., 2019; Lukačišin and Bollenbach, 2019). For example, suppressive interactions have been shown to favor wild type (i.e., drug-sensitive strains) over drug-resistant

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Box 1. Definitions of important terms used

COMBINATION TYPES

Higher-order combination: a drug combination of three or more drugs

Lower-order combination: a drug combination consisting of a smaller number of drugs that are included within a higher-order combination; in a 5-drug combination all combinations with four of those drugs, all combinations with three of those drugs, and all combinations of two of those drugs within the 5-drug combination are considered to be a lower-order combination to that specific 5-drug combination

DRUG INTERACTIONS

Additive interaction: no interaction between drugs; under Bliss independence, the combined effect is as expected assuming each drug is acting independently (Bliss, 1939)

Synergistic interaction: interaction between drugs is stronger than expected; drugs in combination are more effective at inhibiting growth than expected under the additive model

Antagonistic interaction: interaction between drugs is weaker than expected; drugs in combination are less effective at inhibiting growth than expected under the additive model

Suppressive interaction: interaction between drugs results in increased bacterial growth compared with the effects of fewer numbers of drugs; drugs in combination are not only less effective at inhibiting growth than expected under the additive model but also increases growth compared with lower-order combinations or single drugs

Net suppression: a suppressive interaction that occurs between the combination of drugs and the single-drug effects; there is greater bacterial growth when exposed to a drug combination than when exposed to a single drug

Emergent suppression: a suppressive interaction that occurs solely because all drugs are present in the combination

Hidden suppression: a suppressive interaction that occurs between the combination of drugs and a lower-order combination

OTHER USEFUL TERMS

Full-factorial: a dataset that examines higher-order combinations with all their possible lower-order combinations, single-drug effects, along with positive and negative controls. For example, the full-factorial dataset for a single 5-drug combination includes the effects of the 5-drug combination as well as all possible 4-, 3-, and 2-drug combinations of those five drugs, all single drugs, positive controls, and negative controls.

Structure: the way to describe where interactions (net and hidden) occur within a combination

Path: a unique heterarchical grouping containing one representative of each of all the lower-order combinations within the highest-order combination

Nesting: a special type of structure where suppressive interactions occur when an N-drug combination is suppressive to an (N-1)-drug combination and (N-1)-drug combination is suppressive to an (N-2)-drug combination, which is suppressive to an (N-3)-drug combination; this nesting can continue until you compare a 2-drug combination with a single drug.

strains in direct competition *in vitro*. This suggests that suppressive drug combinations could be used to make levels of drug resistance lower in a bacteria population by decreasing the evolutionary fitness of high-resistant strains (Chait et al., 2007). Other studies have shown that suppressive drug combinations could both decrease the rate at which bacteria adapt and evolve resistance to drugs in combination (Hegreness et al., 2008), as well as decrease the likelihood that resistance evolves from spontaneous mutations (Michel et al., 2008). In addition, mechanisms of suppression are being elucidated. The first mechanism for suppression identified was the nonoptimal regulation of the ribosome, which drives the suppressive nature of protein and DNA synthesis inhibitors (Bollenbach et al., 2009). Chaperone deletions can also consistently promote suppressive interactions between chloramphenicol-nitrofurantoin and trimethoprim-mecillinam (Chevereau and Bollenbach, 2015).

Even with this renewed focus on suppression, there has been a bias against publishing antagonistic and suppressive interactions (Singh and Yeh, 2017). The few papers that have looked for suppressive

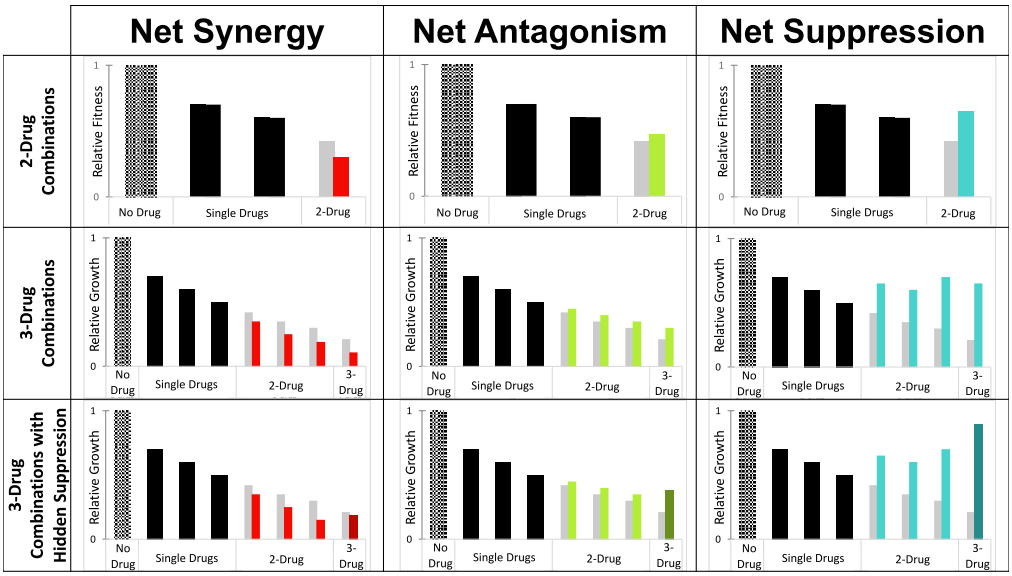

Figure 1. Antibiotic interactions in 2-drug and 3-drug combinations

Hatched bars represent growth in a no-drug environment; black bars represent the fitness of bacteria treated with a single antibiotic. Light gray bars represent the fitness of additive drug interactions, synergistic interactions are in red, antagonistic interactions are in green, and suppressive interactions are in teal. Note that the 2-drug combinations do not need to have the same net interaction type for a 3-drug combination to have a particular net interaction. Suppressive interactions are an extreme form of antagonism: notice that the bacteria treated with the suppressive drug combination has a higher fitness than the single drugs. Importantly, suppressive interactions can be hidden: this occurs when the highest-order combination has higher fitness than a lower-order combination but it does not have higher fitness than any of the single drugs. Thus, hidden suppression can only occur in a combination of 3 or more drugs. Also, note that bacteria treated with the 3-drug combination with hidden suppression has a higher fitness compared with any of the 2-drug combinations but not one of the single drugs.

interactions have found that the amount of suppression varied to some degree but is consistently present in a proportion of drug combinations screened. In drug pairs, the amount of suppression ranges from 5% to 17%. Yeh et al. (2006) reported 8% of the combinations were suppressive. Cokol et al. (2014) reported that 17% were found to be suppressive. This has been considered to be a conservative estimation because the dataset was initially created to identify synergistic combinations (Cokol et al., 2011). Beppler et al. (2017) reported 5% of the 2-drug combinations examined were suppressive. In studies examining higher-order (more than two) drug combinations, suppression rates varied, 3% was observed in Beppler et al. (2017) and 8% was observed in Tekin et al. (2018).

Suppressive interactions in 2-drug combinations are straightforward to identify: bacteria grow better in the presence of two drugs together compared with at least one of the single drugs (Figure 1). Suppressive interactions can also occur in higher-order drug combinations (Figure 1). For example, in a 5-drug combination, five drugs together could have less of an effect than four drugs, or they could have less of an effect than three drugs, two drugs, or a single drug. Also, a 4-drug subset from that five-drug combination could be suppressive to a 3-drug or 2-drug combination, or suppressive to a single drug.

Most studies examine interactions based on deviations from single-drug effects—termed "net suppression" (Box 1). This means the interaction of all the drugs in the combination is determined based only on the comparison with all the single-drug effects (Cokol et al., 2011; Stergiopoulou et al., 2011; Otto-Hanson et al., 2013; Tekin et al., 2017; Katzir et al., 2019). Some studies have also examined emergent interactions and have identified "emergent suppression" (Box 1) or that the effects only from all drugs being in combination are actually suppressive effects (Beppler et al., 2016, 2017; Tekin et al., 2016, 2017, 2018). In

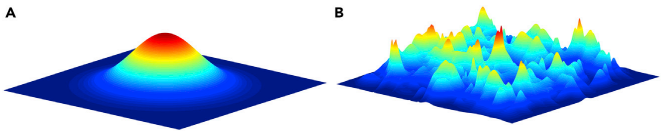

Figure 2. An illustration of the fitness landscapes and the importance of ruggedness in evolutionary trajectories

(A) A smooth landscape only has one peak. As a population evolves to an environment there is always a path that leads to the optimum set of traits resulting in the highest possible fitness.

(B) In a rugged landscape, multiple peaks and valleys make evolving to the highest fitness not as straightforward as in a smooth landscape. Populations may have to cross a valley, which means (1) a loss of fitness must first occur before a net increase in fitness, (2) the population can become stuck at a local peak rather than evolve and ascend to the global peak, or (3) the population must make a jump from one peak to the next. Without the lower-order interactions, we may miss key details of intermediate peaks and valleys in the fitness landscape.

some instances, a suppressive interaction occurs due to suppression relative to a lower-order non-single-drug rather than a single drug; these interactions are termed "hidden" (Beppler et al., 2017; Tekin et al., 2018) (Figure 1). The term "hidden" is used because without examining the lower-order, non-single-drug effects we would never realize there was a suppressive effect (Beppler et al., 2017). For example, when examining a 3-drug combination's effect on bacteria, the interaction of the 3-drug combination is typically compared only with the single-drug effects. Often and not surprisingly, there is increased killing in the 3-drug combination compared with treatment with just one drug. However, the "hidden" part of the interaction comes from the fact that the 3-drug combination could do a worse job at killing bacteria than a subset using two of the three drugs (Figure 1, Box 1). Thus, the phenomenon of hidden interactions means that lower-order combinations are an important part of determining the interaction type of drug combinations.

Why do hidden interactions matter, and why does understanding the structure of these interactions matter? There are important consequences of interactions from both the basic science and the clinical perspectives. From an evolutionary perspective, interactions play an important role in the ecological and evolutionary trajectories of populations. Hidden suppressive interactions are typically not seen in a traditional examination of drug combinations. Considering these hidden interactions would substantially alter the topography of a fitness landscape.

Fitness landscapes are the visualization of the relationships between factors such as stressors or genetic mutations and their effects on fitness (Wright, 1932, 1988). The highest peak in the fitness landscape is the most optimal combination for the bacteria to grow. Although multiple peaks may indicate that there are multiple good combinations of environments for the bacteria to grow, they also create valleys that can be difficult for populations to cross because an individual with intermediate traits or environments between peaks will face an overall decrease in fitness (Figure 2).

From a clinical perspective, using combinations that have hidden suppressive interactions could change the efficacy of a treatment: finding optimal combinations could be thrown off-course by hidden interactions. Understanding the structure and patterns of hidden interactions could therefore be important from both evolutionary and clinical perspectives.

However, studying hidden interactions has been difficult for two primary reasons: first, logistically, there has been substantial difficulty in obtaining full-factorial, higher-order drug interaction measurements. To obtain the full-factorial, growth measurements for all single and all lower-order subsets of drug combinations need to be determined. For example, the full-factorial for a single 5-drug combination includes one 5-drug combination, five 4-drug combinations, ten 3-drug combinations, ten 2-drug combinations, five single drugs alone, and a no-drug control. To obtain data from many full-factorial higher-order combinations has historically been challenging. Second, understanding conceptually and theoretically how to quantify interactions of higher-order drug combinations has been difficult. The logistic difficulty has been alleviated by automated robotics that can handle thousands of measurements in parallel, allowing focused questions that rely on large quantities of measurements. From the conceptual side, we can now accurately categorize the properties of the combination and the interactions, including emergent properties (such as emergent interactions and hidden suppression) (Beppler et al., 2016; Tekin et al., 2016, 2017, 2018). Emergent

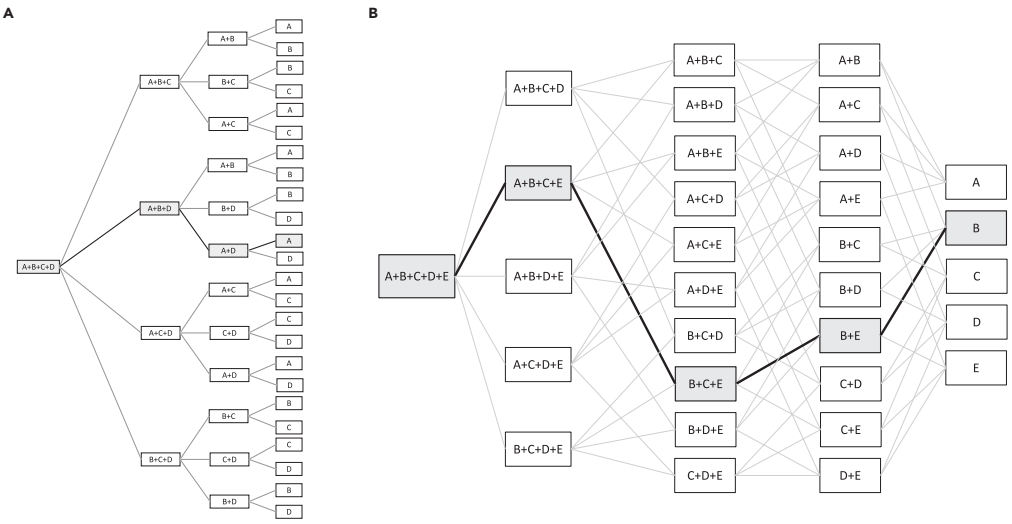

Figure 3. The paths for a 4-drug and a 5-drug combination consisting of drugs A, B, C, D, and E

(A) All 24 possible paths are shown for a 4-drug combination.

(B) All 120 possible paths are shown for a 5-drug combination. For both the 4-drug (A) and 5-drug (B) combinations, a single path is shown in a bold line with the highest-order combination and each lower-order combination highlighted in gray. This single path represents a unique set of drugs, one at each level of combinations (4-drug, 3-drug, 2-drug, and a single drug), allowing for an assessment of any nesting. For this example, nested hidden suppression occurs when the 5-drug combination is suppressive to the 4-drug, the 4-drug combination is suppressive to a 3-drug combination, and the 3-drug combination is then suppressive to a 2-drug combination. And, if appropriate, the 2-drug combination is suppressive to the single-drug effects (this is only considered if the combination is net suppressive). If this is true for all paths the combination is considered to be fully nested. If this is only observed in some paths the combination is considered to be partially nested.

properties are only found in higher-order combinations and are solely because the combination is higher order, that is, the interaction occurs only due to all drugs combining and not due to lower-ordered combinations.

Here we propose a systematic examination of the structure of suppressive interactions (net, emergent, and hidden) in higher-order drug combinations. Specifically, we ask (1) how prevalent are hidden suppressive interactions? (2) What is the structure of a suppressive interaction: are they likely to be suppressive to the next lower-order combination? For example, do we primarily see a 5-drug combination that is suppressive relative to a 4-drug combination, or are there larger jumps in suppression, for example, a 5-drug combination that is suppressive relative to a 2-drug combination? Or is suppression likely to be nested—that is, if a 5-drug combination is suppressive, is it likely to be suppressive to 4-drug and 3-drug subsets within the 5-drug combination? (3) Lastly, are some antibiotics or main mechanism of actions more likely to be involved in general suppressive interactions?

RESULTS

We re-examined the dataset collected and published in Tekin et al. (2018) to examine the presence and patterns of suppressive interactions (both hidden and net) within these combinations. A summary of methods used in Tekin et al. (2018) is provided in the *Transparent methods* section of the supplemental information. We compared the fitness of the highest-order interaction with all lower-order interactions, to determine if hidden suppression was present within the combination. This information was then examined through the use of paths. A path is a unique heterarchical grouping containing one representative of each of all the lower-order combinations within the highest-order combination (Figure 3). We use these paths to identify what suppressive interactions occur within a combination and to detect nesting of hidden suppression. That is, for example, "full" nesting occurs in a 5-drug combination when the 5-drug

Table 1. A list of the names, concentrations, main mechanism of action, mean relative growth compared with a no-drug control, and the abbreviation of the antibiotics used in this study

Name (Abbreviation)	Main Mechanism of Action	Concentration (μM)	Relative Growth (%)	Standard Error (%)
Ampicillin (AMP)	Cell wall	1–2.89	1–77.43	1–3.05
		2–2.52	2–86.01	2–1.74
		3–1.87	3–87.06	3–2.42
Cefoxitin sodium salt (FOX)	Cell wall	1–1.78	1–83.46	1–4.73
		2–1.37	2–92.13	2–2.58
		3–0.78	3–93.33	3–1.81
Trimethoprim (TMP)	Folic acid biosynthesis	1–0.22	1–79.59	1–3.89
		2–0.15	2–74.63	2–4.26
		3–0.07	3–68.20	3–3.93
Ciprofloxacin hydrochloride (CPR)	DNA gyrase	1–0.03	1–92.14	1–1.69
		2–0.02	2–92.14	2–2.40
		3–0.01	3–91.06	3–2.17
Streptomycin (STR)	Aminoglycoside Ribosome, 30S	1–19.04	1–81.10	1–6.50
		2–16.6	2–90.77	2–1.37
		3–12.25	3–83.53	3–4.30
Doxycycline hyclate (DOX)	Ribosome, 50S	1–0.35	1–75.15	1–5.51
		2–0.27	2–76.53	2–5.13
		3–0.15	3–70.01	3–4.73
Erythromycin (ERY)	Ribosome, 50S	1–16.62	1–84.25	1–5.77
		2–8.29	2–84.29	2–5.60
		3–1.78	3–79.63	3–5.91
Fusidic acid sodium salt (FUS)	Ribosome, 30S	1–94.42	1–82.31	1–2.51
		2–71.01	2–78.82	2–2.83
		3–37.85	3–82.62	3–2.47

combination (A + B + C + D + E) is suppressive to a 4-drug combination (A + B + D + E) and that 4-drug combination is suppressive to a 3-drug combination (A + D + E), which is then suppressive to a 2-drug combination (A + D). Analyzing paths will enable us to understand the structure of the interactions—determining which comparisons between a specific lower-order combination and the highest-order combination are suppressive (Box 1). For a fuller description of the rationale behind the use of paths, please see the transparent methods section of the supplemental information. Please note that when referring to a single drug the full name of the antibiotic is written out, and when referring to a combination as a single entity the abbreviations of the drugs (Table 1) within the combination are used. For example, a combination containing the drugs ampicillin, fusidic acid, and streptomycin is listed as AMP + FUS + STR.

The prevalence of hidden suppression

Nearly all higher-order combinations of unique drugs had at least one dose that produced a hidden suppressive interaction. Out of all the possible 182 higher-order drug combinations (fifty-six 3-drug combinations + seventy 4-drug combinations + fifty-six 5-drug combinations) only five (four 3-drug combinations and one 5-drug combination) had no unique dose that had hidden suppression: AMP + FUS + ERY, AMP + FOX + FUS, FOX + CRP + FUS, STR + FOX + FUS, and TMP + STR + FUS + DOX. Among all 20,790 of unique drug-dose combinations studied, suppressive interactions are observed in 54% (11,302) of combinations. With only 17% (3,534) of the total combinations identified as net suppressive (Tekin et al., 2018), the remaining 7,768 combinations with suppressive interactions only contain hidden suppressive interactions. By solely considering the highest-order combination and the single-drug effects, 69% of the combinations with suppressive interactions would not be identified (i.e., 7,768 out of 11,302). As the number of drugs in a unique drug dosage combination increases so does the percentage of combinations with hidden suppression: 33% of the 3-drug combinations, 48% of the 4-drug combinations, and 59% of the 5-drugs combinations had hidden suppression (Figure 4).

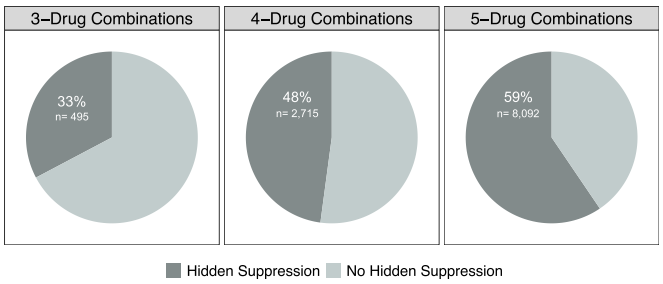

Figure 4. Hidden suppression is present in a majority of higher-order combinations

Hidden suppression was found in all levels examined—3-drug, 4-drug, and 5-drug combinations. The amount of hidden suppression increases as the number of drug increases.

In cases where the net interaction is synergistic or additive, hidden suppression can still occur when the highest-order combination is compared with a lower-order combination (Figures 1, S1, and S2). Importantly, for a combination to contain hidden suppression, it is not dependent on the interaction type based on comparing the fitness values to the single drugs alone. For instance, a synergistic 4-drug combination that results in 20% relative fitness compared with no-drug environments can have a lower-order synergistic 2-drug combination that results in 10% relative fitness. This example then also has hidden suppression because the 4-drug combination results in more bacterial growth than the lower-order 2-drug combination but is still below the additive effects of the single drugs (Figure 1). Net additive combinations had hidden suppression in 27% of 3-drug combinations, 40% in 4-drug combinations, and 67% in 5-drug combinations (Figure 5). In net synergistic combinations, hidden suppression was found in 0% of 3-drug combinations, 7% of 4-drug combinations, and 23% in 5-drug combinations (Figure 5). Hidden suppression in net antagonistic combinations also increased as the number of drugs increased: 52% of 3-drug combinations, 71% of 4-drug combinations, and 72% in 5-drug combinations. In contrast, combinations that are net suppressive showed a decrease in the amount of hidden suppression as the number of drugs increased: 96% of 3-drug combinations, 92% of 4-drug combinations, and 88% in 5-drug combinations. These trends—the increase in the amounts of hidden suppression in synergistic, additive, and antagonistic with the increase in the number of drugs and the decrease in hidden suppression with the increase in the number of drugs among the suppressive interactions—are also observed when examining emergent interactions (Figure 5).

The structure of hidden suppression

When addressing the structure of hidden suppression, it is important to recognize that in each drug combination multiple lower-order interactions are occurring. For example, in a 3-drug combination, there are three unique 2-drug combinations within it. Using the same framework, in a 5-drug combination there are ten unique 2-drug combinations, ten unique 3-drug combinations, and five unique 4-drug combinations. This results in a total of 25 possible hidden interactions. Combinations that contain hidden suppressive interactions can have suppressive interactions with one of the 25 possibilities, all of them, or any amount in between.

The highest-order combination has *N* drugs and is compared with all of the lower-order combinations to see where hidden suppression took place (Table 2). When comparing net suppressive combinations and those that only have hidden suppression, there are more instances of hidden suppression in combinations that are net suppressive no matter the number of drugs in the lower-order combination (Table 2, Figure 6). For example, in a 4-drug combination, there is suppression to the 3-drug combinations in 71% in net suppressive combinations, whereas in combinations with only hidden suppression it was only observed 60% of the time. Combinations that are net suppressive also have the highest amounts of hidden suppression occurring between all possible lower-order combinations (Figure 6). For example, in a 5-drug combination, there are a total of ten possible 2-drug combinations. In net suppressive 5-drug combinations, hidden suppression occurs between the highest-order combination and all possible 2-drug combinations roughly 60% of the time. This occurs in less than 20% of 5-drug combinations that only have hidden suppression. This

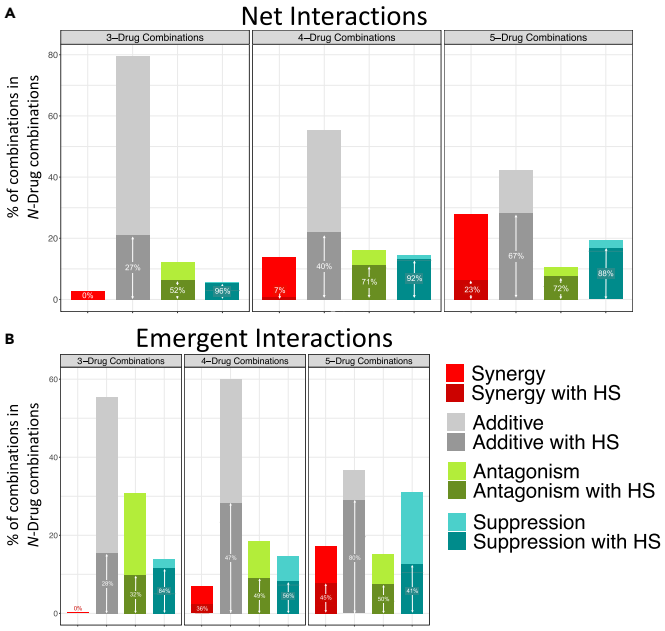

Figure 5. The distributions and relative proportion of hidden suppression for each interaction type for net (A) and emergent (B) interactions for 3-, 4-, and 5- drug combinations. The proportion of combinations with hidden suppression (HS) of suppressive interactions (teal) decreases as the number of drugs in a combination increases. The percentage written inside the darker shades of the bars represents the proportion of combinations with hidden suppression present in that specific interaction type. The y axis is the percentage of each interaction type within the designated level of the drug combination, showing the overall distribution of net or emergent interactions. For example, in (A) the net suppressive 4-drug combinations, 92% of the combinations have hidden suppression within them. As the number of drugs increases, the amount of hidden suppression within additive, synergistic, and antagonistic combinations also increase.

trend, of hidden suppression being more common in net suppressive combinations than only hidden suppression combinations, can be observed no matter how many drugs are in the highest-order combination or the number of drugs in the lower-order combination it is being compared to. It also strengthens as the number of drugs in the highest-order combination increases. Figure 6 compares the amounts of hidden

Table 2. Net suppressive combinations have more hidden suppression than combinations that are *not* net suppressive

Hidden Suppression Found Between	Hidden Suppression Only (%)	Net Suppression (%)
5-Drugs versus 4-drugs	53	80
5-Drugs versus 3-drugs	41	79
5-Drugs versus 2-drugs	40	80
4-Drugs versus 3-drugs	60	71
4-Drugs versus 2-drugs	61	75
3-Drugs versus 2-drugs	76%	77%

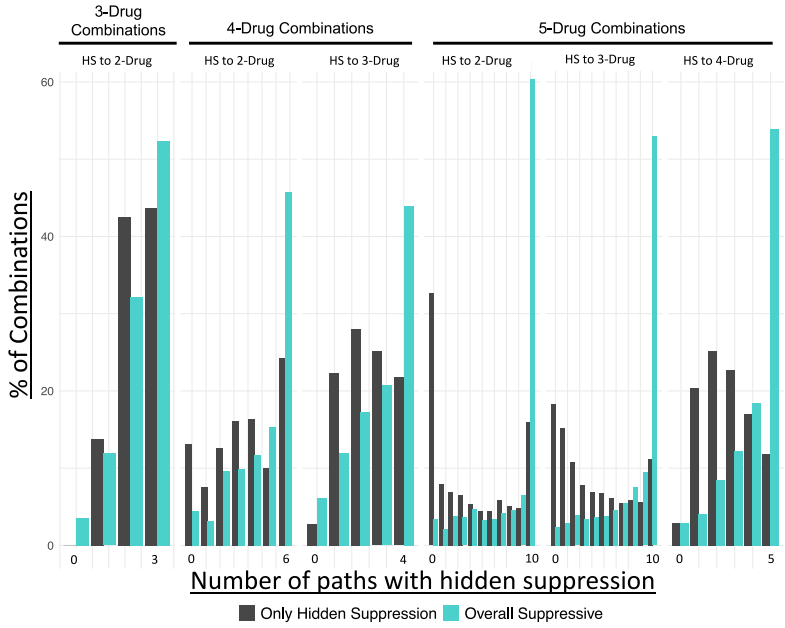

Figure 6. Hidden suppressive interactions occur more frequently within net suppressive combinations rather than within non-net suppressive combinations

The amounts of hidden suppression are shown out of the total number of lower-ordered combinations within a single higher-order combination that is either net suppressive (teal) or has some instances of hidden suppression (gray).

suppression in net suppressive combinations and only hidden suppression combinations. Overall, the difference between net suppressive combinations and only hidden suppression combinations is smaller in 3-drug combinations than in 5-drug combinations. This is especially true when observing if there is hidden suppression for all possible options of *N*-drugs in a lower-order combination.

For net suppressive combinations, full nesting—when fitness at any order is greater than the fitness of the next lower-order combination in all paths including single-drug effects—was only observed in the 3-drug and 4-drug combinations. A majority of net suppressive combinations were considered to have net suppression, wherein at least one path, the fitness at any order must be greater than the fitness of all lower-orders (defined in the transparent methods, Table S1) (Figure S3). When examining the potential nesting of non-net suppressive combination, single-drug effects do not need to be considered because by definition there would be no suppression to the single drugs. All net synergistic combinations only contain hidden suppression that does not fall into any special case.

Likelihood of specific drugs or mechanisms of action involved in suppressive interactions

We used logistic regressions to determine if any drug or the main mechanism of action may have a positive association with general suppressive interactions (hidden and net). The presence of trimethoprim alone was found to be significantly positively associated with suppressive interactions for 3-drug, 4-drug, and 5-drug combinations (Tables 3, S2, and S6). Ciprofloxacin, doxycycline, and erythromycin only had a significant positive association with suppressive interactions in 4-drug and 5-drug combinations (Tables S2 and S6). The presence of trimethoprim increased the odds of a 3-drug, 4-drug, and 5-drug combination being suppressive by roughly 2-fold ($p < 0.001$). The combined presence of ciprofloxacin and trimethoprim (CPR + TMP) and cefoxitin and trimethoprim (FOX + TMP) were also found to significantly increase the

Table 3. Logistic regression of a single drug with 3-drug combinations with some levels of suppressive interactions (hidden and net)

		Confidence Interval				
Term	Coefficient	0.30%	99.70%	p Value	Odds Ratio	Probability (%)
AMP	−0.416	−0.720	−0.117	1.58E-04	0.660	40
CPR	0.019	−0.277	0.311	0.863	1.019	50
DOX	0.096	−0.198	0.388	0.371	1.100	52
ERY	−0.112	−0.409	0.183	0.302	0.894	47
FOX	−0.085	−0.382	0.208	0.429	0.918	48
FUS	−0.868	−1.185	−0.560	2.96E-14	0.420	30
STR	−1.684	−2.053	−1.337	5.02E-38	0.186	16
TMP	**0.729**	**0.443**	**1.018**	**3.75E-12**	**2.074**	**67**
AIC: 1678.2	Bonferroni- corrected α: 0.00625				Degrees of Freedom: 1512	

Terms in **bold** have a significant positive association with suppressive interactions.

probability of finding suppressive interactions in 3-drug, 4-drug, and 5-drug combinations ($p < 0.001$) (Tables 4, S3, and S7). The combined presence of ampicillin and ciprofloxacin, ciprofloxacin and erythromycin, doxycycline and cefoxitin, and erythromycin and trimethoprim had a positive association with suppressive interactions for 4-drug and 5-drug combinations ($p < 0.001$) (Tables S3 and S7).

When considering the main mechanism of action rather than individual antibiotics, the presence of the antibiotic acting on folic acid biosynthesis (trimethoprim) was found to be significantly positively associated with suppressive interactions ($p < 0.01$) in 3-drug, 4-drug, and 5-drug combinations (Tables 5, S4, and S8). There were only two positive associations that occur across all levels of higher-order drug combinations (i.e., 3-drugs, 4-drug, and 5-drug combinations): they are with the antibiotic acting on folic acid biosynthesis, trimethoprim, alone ($p < 0.0001$) and the combination of two main mechanism of actions—folic acid biosynthesis and the DNA gyrase ($p < 0.001$) (Tables 6, S5, and S9). The probability of a combination having suppressive interactions decreases with the presence of an antibiotic acting on the 30S ribosomal subunit alone in the 3-drug, 4-drug, and 5-drug combinations ($p < 0.0001$) (Tables 5, S4, and S8).

DISCUSSION

Although it was previously reported that higher-order drug combinations had a substantial amount of suppressive interactions (14% in Beppler et al. (2017) and 8% in Tekin et al. (2018)), there has been no further work on understanding the patterns and prevalence of higher-order suppressive interactions, particularly hidden interactions. The idea of hidden suppressive interactions was first introduced by Beppler and colleagues several years ago (Beppler et al., 2017). New technologies are now allowing rapid detection of suppressive interactions using both very small volumes of bacterial culture and antibiotic combinations (<1uL) and very short time frames of several hours (Cokol et al., 2011, 2014; Churski et al., 2012). New conceptual advances allow us to examine higher-order interactions and emergent properties of drug combinations (Beppler et al., 2016; Tekin et al., 2016; Katzir et al., 2019; Lukačišin and Bollenbach, 2019). Because of this, suppressive interactions have received more focus recently (see review Singh and Yeh (2017)). We have shown that even with recent advancements and interest in suppression, one can severely underestimate the number of suppressive interactions by not considering hidden suppression.

When examining hidden suppression, increasing the number of drugs in a combination also increases the number of possible lower-order combinations, thus possibly increasing the total number of combinations with hidden suppression interaction. When we look at the overall percentage of combinations with hidden suppression, this value steadily increases from 33% to 48%–59% as the number of drugs increases (Figure 4). This would explain the trends we see in Figure 5 for synergistic, additive, and antagonistic combinations. However, this does not offer a viable explanation for the negative correlation between the amount of hidden suppression and the number of drugs in a combination of net and emergent suppressive combinations.

Table 4. Logistic regression of pairwise drugs with 3-drug combinations with some levels of suppressive interactions (hidden and net)

		Confidence Interval				
Term	Coefficient	0.10%	99.90%	p Value	Odds Ratio	Probability (%)
AMP + CPR	0.126	−0.576	0.817	0.571	1.134	53
AMP + DOX	0.252	−0.379	0.877	0.208	1.287	56
AMP + ERY	−0.778	−1.498	−0.097	4.95E-04	0.460	31
AMP + FOX	−0.524	−1.291	0.212	0.029	0.592	37
AMP + FUS	−1.671	−2.593	−0.861	1.27E-09	0.188	16
AMP + STR	−1.432	−2.477	−0.559	2.23E-06	0.239	19
AMP + TMP	**0.953**	**0.305**	**1.627**	**6.08E-06**	**2.594**	**72**
CPR + DOX	0.159	−0.439	0.753	0.403	1.172	54
CPR + ERY	−0.208	−0.836	0.406	0.294	0.812	45
CPR + FOX	−0.857	−1.565	−0.182	1.01E-04	0.425	30
CPR + FUS	−0.307	−1.008	0.359	0.159	0.736	42
CPR + STR	−0.755	−1.561	−0.027	1.94E-03	0.470	32
CPR + TMP	**0.739**	**0.122**	**1.379**	**2.25E-04**	**2.094**	**68**
DOX + ERY	−0.189	−0.798	0.407	0.326	0.828	45
DOX + FOX	0.570	−0.039	1.185	3.51E-03	1.768	64
DOX + FUS	0.388	−0.238	1.002	0.050	1.474	60
DOX + STR	−0.939	−1.783	−0.186	2.10E-04	0.391	28
DOX + TMP	−1.044	−1.685	−0.420	2.31E-07	0.352	26
ERY + FOX	0.182	−0.485	0.846	0.392	1.199	55
ERY + FUS	−0.775	−1.498	−0.101	4.93E-04	0.461	32
ERY + STR	0.030	−0.682	0.699	0.890	1.031	51
ERY + TMP	0.464	−0.155	1.094	0.020	1.590	61
FOX + FUS	−0.848	−1.632	−0.122	4.23E-04	0.428	30
FOX + STR	−1.607	−2.635	−0.740	8.27E-08	0.201	17
FOX + TMP	**1.026**	**0.387**	**1.698**	**9.05E-07**	**2.790**	**74**
FUS + STR	−0.942	−1.924	−0.104	1.06E-03	0.390	28
FUS + TMP	−0.058	−0.688	0.559	0.769	0.943	49
STR + TMP	−0.978	−1.756	−0.259	4.08E-05	0.376	27
AIC: 1579.7	Bonferroni-corrected α: 0.00179				Degrees of Freedom: 1512	

Terms in **bold** have a significant positive association with suppressive interactions.

In 2-drug combinations, it has been shown that a combination of DNA synthesis inhibitors and protein synthesis inhibitors has higher amounts of suppression (Yeh et al., 2006; Chait et al., 2007; Bollenbach et al., 2009). Thus, we expected that we might find some drugs or main mechanisms of actions more consistently involved in suppressive interactions, and this was indeed the case. We have shown that there is a significant positive association with suppressive interactions and interference with the 50S ribosomal subunit in combination with a DNA gyrase in 4-drug combinations and a significant positive association with suppressive interactions and interference with the 30S ribosomal subunit in combination with a DNA gyrase in 5-drug combinations. These findings are supported by the one suppressive mechanism that is very well understood (Bollenbach et al. (2009).

The main mechanism of action is one way that antibiotics are commonly grouped. We expected to see similar patterns of association between the logistic regressions based on specific drugs and the main mechanism of actions. We observe this similarity with the main mechanism of actions affecting folic acid biosynthesis trimethoprim, affecting the 50S ribosomal subunit—doxycycline and erythromycin and

Table 5. Logistic regression of the main mechanism of actions with 3-drug combinations with some levels of suppressive interactions (hidden and net)

		Confidence Interval				
Term	Coefficient	0.50%	99.50%	p Value	Odds Ratio	Probability (%)
Cell wall	−0.267	−0.517	−0.018	5.76E-03	0.765	43
Folic acid biosynthesis	**0.742**	**0.470**	**1.017**	**2.74E-12**	**2.100**	**68**
DNA gyrase	0.035	−0.246	0.314	0.747	1.036	51
Ribosome, 30S	−1.462	−1.719	−1.212	5.36E-50	0.232	19
Ribosome, 50S	0.062	−0.188	0.312	0.524	1.064	52
AIC: 1716	Bonferroni-corrected α: 0.01			Degrees of Freedom: 1512		

Terms in **bold** have a significant positive association with suppressive interactions.

affecting DNA gyrase—ciprofloxacin. As previously described, the identification of DNA gyrases and protein synthesis can be expected to be positively associated with suppressive interactions. However, folic acid biosynthesis interference is positively associated with suppressive interactions in all levels of drug combinations (3-drug, 4-drug, and 5-drug combinations). We suggest that this cellular mechanism may also be a mechanism for suppression and could be a fruitful avenue for future studies.

Hidden suppressive interactions can affect fitness landscapes, which means they ultimately could affect the evolutionary trajectory of populations. For example, if we use a drug combination with a corresponding fitness landscape based only on information from the single drugs and the 5-drug combination, we could end up with a landscape topography that looks very different from a fitness landscape where we had information from all lower-order drugs (Figure 7). This is not surprising because we have more information in the latter than the former. Qualitatively, the fitness landscapes are similar, but there are quantitative differences (Sanchez-Gorostiaga et al., 2019). In contrast, in cases where hidden suppression is present, a landscape without the lower-order interaction information would look very different from a landscape with all the lower-order interactions (Figures 7 and S4). Qualitatively, there are important differences between the fitness landscape because there are local valleys and peaks that are present in the latter and not present in the former. These valleys and peaks can affect how a population evolves and where it ends up (Østman et al., 2011; Palmer et al., 2015; Bendixsen et al., 2017).

Within a specific drug pair, recent work has shown that the concentrations at which two drugs veer into suppressive territory (from, for example, additivity) could be understood via a cost-benefit analysis. There is a trade-off between a drug inducing resistance (good for the bacterial cell) and increasing toxicity (bad for the bacterial cell), and this trade-off could explain why certain concentrations in one drug pair are suppressive, whereas other concentrations exhibit different interaction types (Wood and Cluzel, 2012). Furthermore, with some exceptions, suppressive interactions, as with most interactions, are typically robust to genetic mutations (Chevereau and Bollenbach, 2015).

Clinicians traditionally favor treatments with synergistic combinations, because it limits the number of antibiotics prescribed to the patient limiting any potential adverse effects (Lepper and Dowling, 1951; French et al., 1985; Sun et al., 2013; Arya et al., 2019), rather than treatment with suppressive combinations. This is because by definition, using suppressive interactions means using higher drug concentrations to achieve the same bacterial killing effect as drugs that are additive or synergistic. Thus, hidden suppressive interactions are ones that could be confounding in the clinic. As more treatments move to higher-order combinations of drugs (Mbuagbaw et al., 2016; Sun et al., 2016; Morimoto et al., 2018; Tsigelny, 2019), it becomes critical to understand where suppressive interactions may be hidden, to avoid surprising and unwelcome clinical outcomes. For example, as shown in Figure 7, if one were to use a combination of CPR + ERY + STR + FUS + TMP and if we only compared the results of the five drugs together with all the single drugs alone, we would think this was a potentially useful combination, in that killing efficiency seems to increase relative to the five single drugs by themselves. But once we examine these in light of emergent properties, what we see is that CPR + ERY + STR + FUS + TMP has a lower killing efficiency than CPR + STR + FUS + TMP.

Table 6. Logistic regression of the pairwise main mechanism of actions with 3-drug combinations with some levels of suppressive interactions (hidden and net)

		Confidence Interval				
Term	Coefficient	0.20%	99.80%	p Value	Odds Ratio	Probability (%)
Cell wall + folic acid biosynthesis	**1.016**	**0.548**	**1.495**	**5.52E-10**	**2.762**	**73**
Cell wall + DNA gyrase	−0.417	−0.949	0.096	0.021	0.659	40
Cell wall + ribosome, 30S	−1.565	−2.050	−1.109	6.46E-22	0.209	17
Cell wall + ribosome, 50S	0.265	−0.114	0.645	0.044	1.303	57
Folic acid biosynthesis + DNA gyrase	**0.742**	**0.175**	**1.326**	**1.90E-04**	**2.100**	**68**
Folic acid biosynthesis + ribosome, 30S	−0.304	−0.790	0.172	0.068	0.738	42
Folic acid biosynthesis + ribosome, 50S	−0.522	−1.022	−0.038	2.10E-03	0.593	37
DNA gyrase + ribosome, ribosome, 30S	−0.529	−1.082	−0.009	4.25E-03	0.589	37
DNA gyrase + ribosome, ribosome, 50S	−0.039	−0.507	0.428	0.808	0.961	49
Ribosome, 30S + ribosome, 50S	−0.160	−0.572	0.252	0.262	0.852	46
Cell wall + cell wall	−0.584	−1.148	−0.044	2.18E-03	0.558	36
Ribosome, 30S + ribosome, 30S	−1.565	−2.411	−0.857	3.93E-09	0.209	17
Ribosome, 50S + ribosome, 50S	−0.402	−0.905	0.085	0.019	0.669	40
AIC: 1670.9	Bonferroni-corrected α: 0.0039			Degrees of Freedom: 1512		

Terms in **bold** have a significant positive association with suppressive interactions.

In conclusion, we show here that higher-order drug combinations exhibit a large number of suppressive interactions, and these interactions are primarily hidden. That is, we would never know there was a suppressive interaction if we only looked at the effects of the highest-order combinations and compared that with all the single-drug effects. Uncovering hidden suppressive interactions could decrease surprises regarding how populations evolve to drug combinations. At the same time, identifying hidden suppression can yield valuable information about underlying reasons regarding which drug combinations could be useful and which ones should be avoided.

Limitations of the study

Here we exemplify the need to consider hidden interactions and the possible implications of hidden suppression. To do this we examined an extensive dataset and found intriguing results. However, ideally, additional data could be analyzed with an even larger group of drugs examined, allowing for multiple representatives from each antibiotic class and the main mechanism of actions. The dataset from Tekin et al. (2018) used low levels of inhibition for each individual drug in an attempt to have detectable growth when antibiotics are used in 5-drug combinations. The low inhibition of each individual drug can affect the fraction of net-suppressive interactions by narrowing the range of a suppressive interaction. But ultimately these concentrations were chosen to avoid killing off the entire bacterial populations before a 5-drug combination could be examined. Finally, future studies of drug interactions can incorporate bootstrapping and other methods to determine robustness of results.

Resource availability

Lead contact

Dr. Pamela Yeh, PhD holds the role of lead contact and can be reached at pamelayeh@ucla.edu

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data and code has been made freely available via Mendeley Data (https://data.mendeley.com/datasets/ts2hnd72yf/1).

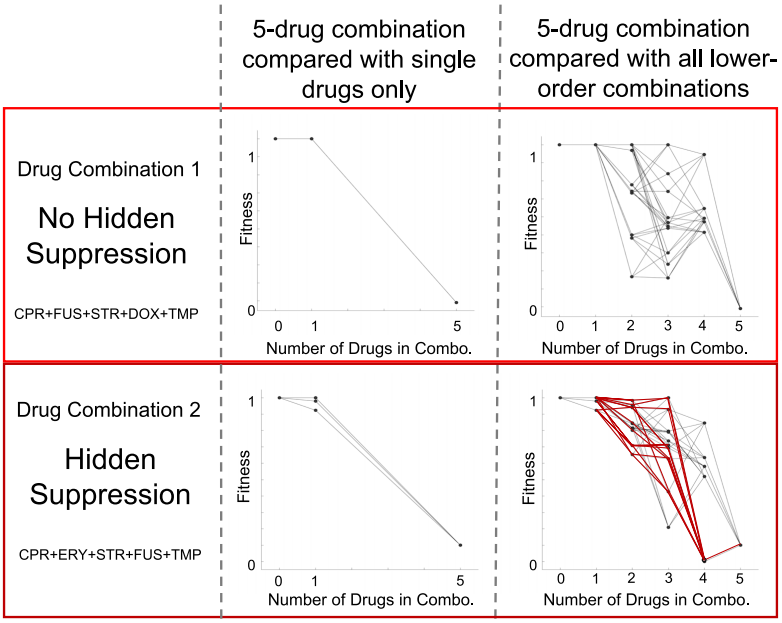

Figure 7. Fitness graphs show the importance of considering hidden interactions

Fitness graphs show similar information as a fitness landscape; they both help to visualize the relationships between stressors or genetic mutations and their effects on fitness. However, fitness graphs can be more appropriate for discrete data. Here we show fitness graphs of two synergistic 5-drug combinations (for abbreviations see Table 1). Drug combination 1 has no hidden suppression (top), and drug combination 2 has hidden suppression (bottom). The left-hand side shows the fitness graphs not considering the hidden suppression; notice how similar these two appear to be. Although the figures on the right-hand side show the fitness graphs including the lower-order combinations, notice the increase in ruggedness is due to the hidden suppressive interactions (the decrease in fitness at one of the 4-drug combinations) in the bottom right. The edges in red highlight the paths involved in hidden suppression. For more detailed information about these paths please see Figure S4.

METHODS

All methods can be found in the accompanying transparent methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102355.

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AUTHOR CONTRIBUTIONS

Conceptualization: P.J.Y.; Methodology: P.J.Y., E.T., N.A.L.H., and A.Z.; Analysis: N.A.L.H., A.Z., and M.L.C.; Writing: P.J.Y, N.A.L.H., A.Z., B.Ø., E.T., M.C.L., and S.B.; Supervision: P.J.Y.; Funding Acquisition: P.J.Y.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental information

Hidden suppressive interactions are common in higher-order drug combinations

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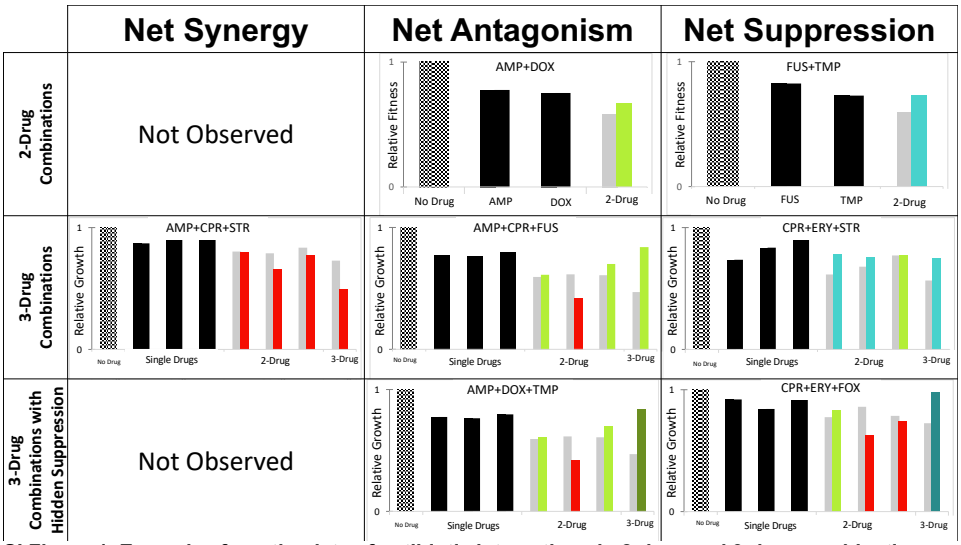
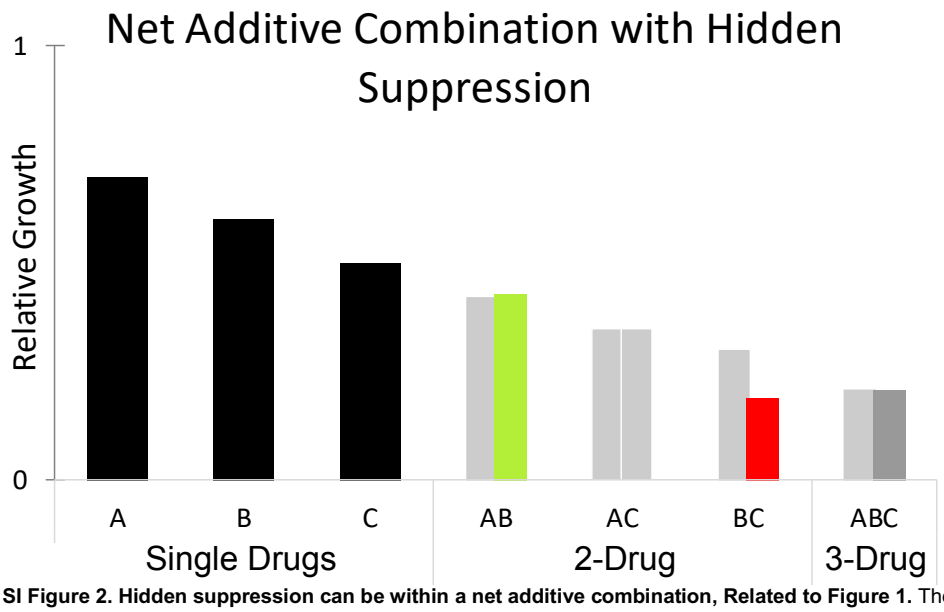
Supplemental Information

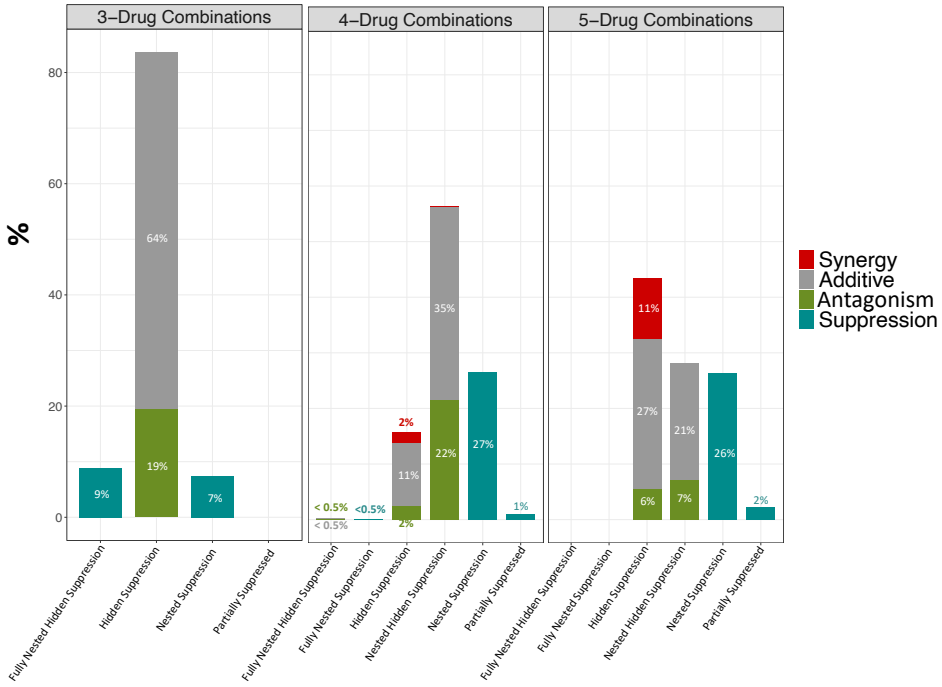
Supplemental Figures



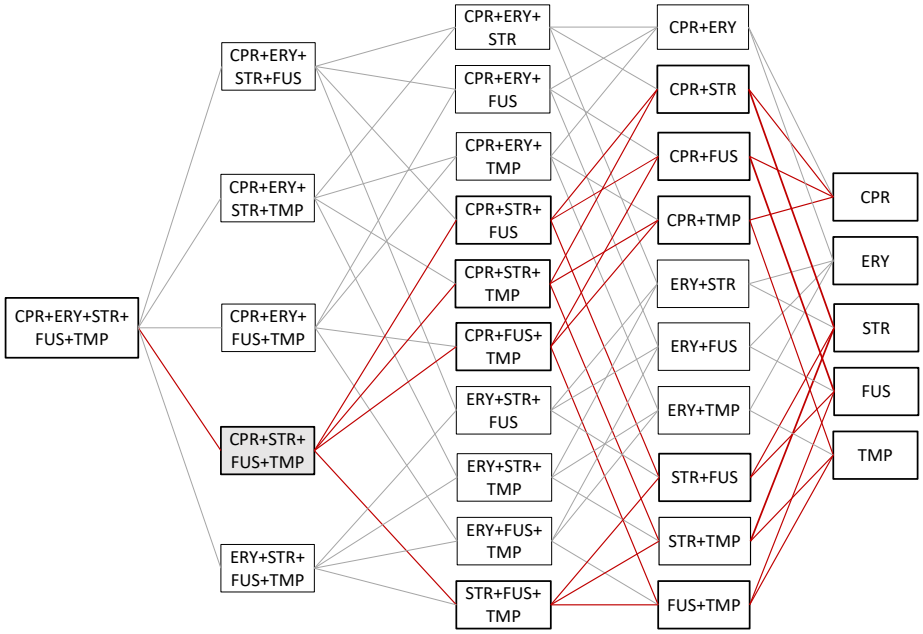
SI Figure 1. Examples from the data of antibiotic interactions in 2-drug and 3-drug combinations, Related to Figure 1. Combinations are listed above bar graphs for each example (for abbreviations see Table 1). Hatched bars represent growth in a no-drug environment, black bars represent the fitness of bacteria treated with a single antibiotic. Light gray bars represent the fitness of additive drug interactions, synergistic interactions are in red, antagonistic interactions are in green and suppressive interactions are in teal. Note that the 2-drug combinations do not need to have the same net interaction type for a 3-drug combination to have a particular net interaction. Suppressive interactions are an extreme form of antagonism: notice that the bacteria treated with the suppressive drug combination has a higher fitness than the single drugs. Importantly, suppressive interactions can also be hidden when the highest-order combination has higher fitness than a lower-order combination and not the single drugs. Thus, hidden suppression can only occur in a combination of 3 or more drugs. Also note, that bacteria treated with the 3-drug combination with hidden suppression has a higher fitness compared to any of the 2-drug combinations but not one of the single drugs.



SI Figure 2. Hidden suppression can be within a net additive combination, Related to Figure 1. The bars in black show the effects of the single drugs. The grey bars on the left show the additive expectations given the single drug effects while the bar on the right shows the actual relative growth when exposed to the combination. The 2-drug combinations have varying interactions, combination AB is an antagonistic interaction (green bar), combination AC is an additive interaction so the expected grey bar is the same as the relative growth that is observed, and BC is a synergistic combination (red bar). Due to the nature of a hidden suppressive interaction, a net additive combination can have hidden suppressive interactions (3-drug combination in dark gray) as long as at least one of the lower-order interactions is synergistic (2-drug combination BC in red). Note that although the three-drug combination (dark gray) has the same value as the strictly additive case (light gray) it is considered to have hidden suppression because one of the lower-order 2-drug combinations is synergistic (red). This makes the 3-drug combination have higher fitness than the 2-drug lower-order combination.



SI Figure 3. Distribution of special cases of hidden suppression structure, Related to Figure 5. All net synergistic combinations only have hidden suppression that does not adhere to any special case. 3-drug combinations were only tested for fully hidden suppression, hidden suppression, nested suppression, and partially suppressed, because all other special cases are trivial in a 3-drug combination.



SI Figure 4. Paths of example synergistic drug combination with hidden suppression, Related to Figure 7. All 120 paths for the combination CPR+ERY+STR+FUS+TMP (for abbreviations see Table 1). Paths highlighted in red with bold edges contain hidden suppression between the 5-drug combination and the 4-drug combination CPR+STR+FUS+TMP (shaded in grey). These highlighted paths are the same paths shown in Figure 7.

Supplemental Tables

SI Table 1. Special Case Definitions, Related to Figure 3. A description of each special case definition for both net suppressive interactions and not net suppressive interactions.

Net Suppression Classification $(DA_{\mathrm{N}} > 1.3)$		**Hidden Suppression Classification** $\left({}^{w_{\mathrm{N}}}/_{w_{\text{min of lower orders}}} > 1.3\right)$	
Special Case	Definition	Special Case	Definition
Fully Nested Suppression	In all paths, fitness at any order must be greater than the fitness of all lower-orders.	Fully Nested Hidden Suppression	In all paths, fitness at any order must be greater than the fitness of all lower-orders, **excluding** the single drugs.
Partially Nested Suppression	In at least one path, fitness at any order must be greater than the fitness of all lower-orders.	Partially Nested Hidden Suppression	In at least one path, fitness at any order must be greater than the fitness of all lower-orders, **excluding** the single drugs.
Fully Suppressed	In all paths, fitness at the highest-order(w_{N}) is greater than the fitness of all lower-orders.	Fully Hidden Suppression	In all paths, fitness at the highest-order(w_{N}) is greater than the fitness of all lower-orders, **excluding** the single drugs.
Partially Suppressed	Only some paths have the highest-order(w_{N}) fitness greater than all lower-order fitness.	Partially Hidden Suppression	Only some paths have the highest-order(w_{N}) fitness greater than all lower-order fitness, **excluding** the single drugs.
Suppressive Interaction with Hidden Suppression	The highest-order combination does not fulfill any other conditions but still has at least one hidden suppressive interaction.	Hidden Suppressive Interaction	The highest-order combination does not fulfill any above conditions, but still has an element of hidden suppression.
No Hidden Suppression	No paths have the highest-order(w_{N}) fitness greater than lower-order fitness, excluding first-order(w_1).		

SI Table 2. Logistic regression of single drug with 4-drug combinations with some levels of suppressive interactions (hidden and net), Related to Table 3. Terms in **bold** have a significant positive association with suppressive interactions.

Term	Coefficient	Confidence Interval		p-value	Odds Ratio	Probability
		0.30%	99.70%			
AMP	-0.270	-0.414	-0.127	2.50E-07	0.763	43%
CPR	**0.430**	**0.287**	**0.574**	**2.37E-16**	**1.537**	**61%**
DOX	**0.214**	**0.071**	**0.357**	**4.22E-05**	**1.239**	**55%**
ERY	**0.517**	**0.374**	**0.661**	**7.30E-23**	**1.677**	**63%**
FOX	**0.385**	**0.242**	**0.528**	**2.09E-13**	**1.469**	**60%**
FUS	-0.829	-0.976	-0.683	3.75E-54	0.437	30%
STR	-1.333	-1.481	-1.187	2.61E-135	0.264	21%
TMP	**0.799**	**0.655**	**0.944**	**1.11E-51**	**2.223**	**69%**
AIC: 6693.7		Bonferroni-corrected α: 0.00625			Degrees of Freedom: 5670	

SI Table 3. Logistic regression of pairwise drugs with 4-drug combinations with some levels of suppressive interactions (hidden and net), Related to Table 4. Terms in **bold** have a significant positive association with suppressive interactions.

Term	Coefficient	Confidence Interval		p-value	Odds Ratio	Probability
		0.10%	99.90%			
AMP+CPR	**0.329**	**0.036**	**0.622**	**4.65E-04**	**1.389**	**58%**
AMP+DOX	0.039	-0.251	0.328	0.677	1.039	51%
AMP+ERY	-0.548	-0.839	-0.258	3.69E-09	0.578	37%
AMP+FOX	-0.185	-0.477	0.106	0.047	0.831	45%
AMP+FUS	-0.356	-0.653	-0.061	1.71E-04	0.700	41%
AMP+STR	-0.416	-0.716	-0.119	1.36E-05	0.660	40%
AMP+TMP	**0.694**	**0.395**	**0.995**	**5.26E-13**	**2.001**	**67%**
CPR+DOX	**0.622**	**0.327**	**0.920**	**5.87E-11**	**1.863**	**65%**
CPR+ERY	**0.860**	**0.559**	**1.165**	**7.30E-19**	**2.363**	**70%**
CPR+FOX	-0.519	-0.815	-0.224	4.02E-08	0.595	37%
CPR+FUS	-0.520	-0.816	-0.226	3.69E-08	0.595	37%
CPR+STR	-0.652	-0.949	-0.358	5.72E-12	0.521	34%
CPR+TMP	**0.958**	**0.641**	**1.281**	**8.83E-21**	**2.606**	**72%**
DOX+ERY	0.191	-0.101	0.485	0.042	1.211	55%
DOX+FOX	**0.517**	**0.228**	**0.807**	**2.48E-08**	**1.677**	**63%**
DOX+FUS	-0.132	-0.421	0.156	0.153	0.877	47%
DOX+STR	-0.574	-0.868	-0.283	8.63E-10	0.563	36%
DOX+TMP	-0.159	-0.463	0.147	0.104	0.853	46%
ERY+FOX	0.122	-0.172	0.418	0.198	1.129	53%
ERY+FUS	-0.026	-0.314	0.260	0.774	0.974	49%
ERY+STR	-0.098	-0.387	0.189	0.286	0.906	48%
ERY+TMP	**0.724**	**0.413**	**1.041**	**5.59E-13**	**2.063**	**67%**
FOX+FUS	-0.250	-0.540	0.038	6.77E-03	0.779	44%
FOX+STR	-0.012	-0.302	0.276	0.893	0.988	50%
FOX+TMP	**1.204**	**0.897**	**1.518**	**7.50E-34**	**3.334**	**77%**
FUS+STR	-0.054	-0.361	0.252	0.584	0.948	49%
FUS+TMP	-0.497	-0.798	-0.200	2.11E-07	0.608	38%
STR+TMP	-1.087	-1.392	-0.787	2.92E-29	0.337	25%
AIC: 6308.8		Bonferroni-corrected α: 0.00179			Degrees of Freedom: 5670	

SI Table 4. Logistic regression of the main mechanism of actions with 4-drug combinations with some levels of suppressive interactions (hidden and net), Related to Table 5. Terms in **bold** have a significant positive association with suppressive interactions.

Term	Coefficient	Confidence Interval		p-value	Odds Ratio	Probability
		0.50%	99.50%			
Cell Wall	**0.280**	**0.136**	**0.425**	**5.69E-07**	**1.324**	**57%**
Folic Acid Biosynthesis	**0.848**	**0.709**	**0.988**	**1.64E-55**	**2.335**	**70%**
DNA gyrase	**0.493**	**0.355**	**0.632**	**3.68E-20**	**1.637**	**62%**
Ribosome, 30S	-1.706	-1.877	-1.540	1.47E-149	0.182	15%
Ribosome, 50S	**0.613**	**0.468**	**0.760**	**3.23E-27**	**1.847**	**65%**
AIC: 6871.4		Bonferroni-corrected α: 0.01			Degrees of Freedom: 5670	

SI Table 5. Logistic regression of the pairwise main mechanism of actions with 4-drug combinations with some levels of suppressive interactions (hidden and net), Related to Table 6. Terms in **bold** have a significant positive association with suppressive interactions.

Term	Coefficient	Confidence Interval		p-value	Odds Ratio	Probability
		0.20%	99.80%			
Cell Wall+Folic Acid Biosynthesis	**1.112**	**0.796**	**1.433**	**6.62E-24**	**3.040**	**75%**
Cell Wall+DNA gyrase	-0.219	-0.540	0.102	0.049	0.803	45%
Cell Wall+Ribosome, 30S	-0.488	-0.841	-0.131	7.11E-05	0.614	38%
Cell Wall+Ribosome, 50S	0.263	-0.071	0.592	0.022	1.301	57%
Folic Acid Biosynthesis+DNA gyrase	**0.870**	**0.564**	**1.184**	**5.15E-16**	**2.388**	**70%**
Folic Acid Biosynthesis+Ribosome, 30S	-0.737	-1.084	-0.399	5.13E-10	0.479	32%
Folic Acid Biosynthesis+Ribosome, 50S	0.183	-0.160	0.528	0.124	1.201	55%
DNA gyrase+Ribosome, Ribosome, 30S	**-0.418**	-0.769	-0.072	**5.34E-04**	**0.658**	**40%**
DNA gyrase+Ribosome, Ribosome, 50S	**0.782**	**0.459**	**1.109**	**3.76E-12**	**2.185**	**69%**
Ribosome, 30S+Ribosome, 50S	-0.300	-0.641	0.046	0.012	0.741	43%
Cell Wall+Cell Wall	-0.081	-0.288	0.127	0.263	0.923	48%
Ribosome, 30S+Ribosome, 30S	-0.749	-0.971	-0.533	4.70E-23	0.473	32%
Ribosome, 50S+Ribosome, 50S	**0.346**	**0.138**	**0.556**	**1.82E-06**	**1.413**	**59%**
AIC: 6695.6		Bonferroni-corrected α: 0.0039			Degrees of Freedom: 5670	

SI Table 6. Logistic regression of single drug with 5-drug combinations with some levels of suppressive interactions (hidden and net), Related to Table 3. Terms in **bold** have a significant positive association with suppressive interactions.

Term	Coefficient	Confidence Interval		p-value	Odds Ratio	Probability
		0.30%	99.70%			
AMP	0.033	-0.057	0.123	0.317	1.033	51%
CPR	**0.351**	**0.261**	**0.441**	**9.27E-27**	**1.420**	**59%**
DOX	**0.148**	**0.058**	**0.237**	**7.02E-06**	**1.159**	**54%**
ERY	**0.261**	**0.172**	**0.351**	**1.65E-15**	**1.299**	**56%**
FOX	**0.205**	**0.115**	**0.294**	**4.60E-10**	**1.227**	**55%**
FUS	-0.465	-0.556	-0.374	5.03E-44	0.628	39%
STR	-0.292	-0.383	-0.201	1.36E-18	0.747	43%
TMP	**0.572**	**0.482**	**0.661**	**2.25E-68**	**1.771**	**64%**
AIC: 17458		Bonferroni-corrected α: 0.00625			Degrees of Freedom: 13602	

SI Table 7. Logistic regression of pairwise drugs with 5-drug combinations with some levels of suppressive interactions (hidden and net), Related to Table 4. Terms in **bold** have a significant positive association with suppressive interactions.

Term	Coefficient	Confidence Interval		p-value	Odds Ratio	Probability
		0.10%	99.90%			
AMP+CPR	**0.738**	**0.547**	**0.929**	**1.41E-33**	**2.091**	**68%**
AMP+DOX	-0.008	-0.197	0.181	0.891	0.992	50%
AMP+ERY	-0.480	-0.669	-0.292	1.78E-15	0.619	38%
AMP+FOX	-0.065	-0.254	0.124	0.286	0.937	48%
AMP+FUS	**0.298**	**0.097**	**0.500**	**3.75E-06**	**1.347**	**57%**
AMP+STR	-0.131	-0.327	0.066	0.037	0.877	47%
AMP+TMP	-0.041	-0.232	0.149	0.499	0.960	49%
CPR+DOX	0.128	-0.062	0.318	0.035	1.136	53%
CPR+ERY	**0.513**	**0.323**	**0.703**	**3.81E-17**	**1.670**	**63%**
CPR+FOX	-0.337	-0.526	-0.149	2.29E-08	0.714	42%
CPR+FUS	-0.238	-0.434	-0.041	1.60E-04	0.788	44%
CPR+STR	-0.570	-0.766	-0.375	7.58E-20	0.565	36%
CPR+TMP	**0.704**	**0.512**	**0.898**	**4.02E-30**	**2.023**	**67%**
DOX+ERY	**0.266**	**0.076**	**0.457**	**1.31E-05**	**1.305**	**57%**
DOX+FOX	**0.496**	**0.306**	**0.687**	**3.98E-16**	**1.642**	**62%**
DOX+FUS	-0.502	-0.697	-0.307	9.13E-16	0.606	38%
DOX+STR	-0.461	-0.657	-0.266	1.73E-13	0.631	39%
DOX+TMP	**0.740**	**0.548**	**0.933**	**3.89E-33**	**2.095**	**68%**
ERY+FOX	**0.369**	**0.180**	**0.560**	**1.28E-09**	**1.447**	**59%**
ERY+FUS	-0.483	-0.678	-0.289	7.90E-15	0.617	38%
ERY+STR	-0.183	-0.378	0.012	3.45E-03	0.833	45%
ERY+TMP	**0.821**	**0.629**	**1.015**	**2.54E-40**	**2.274**	**69%**
FOX+FUS	-0.609	-0.803	-0.415	1.19E-22	0.544	35%
FOX+STR	**0.466**	**0.267**	**0.666**	**2.80E-13**	**1.594**	**61%**
FOX+TMP	**0.315**	**0.124**	**0.507**	**2.59E-07**	**1.371**	**58%**
FUS+STR	**1.174**	**0.933**	**1.426**	**3.24E-50**	**3.236**	**76%**
FUS+TMP	-0.388	-0.585	-0.193	6.09E-10	0.678	40%
STR+TMP	-0.802	-0.999	-0.607	2.60E-37	0.448	31%
AIC: 17458		Bonferroni-corrected α: 0.00179			Degrees of Freedom: 13602	

SI Table 8. Logistic regression of the main mechanism of actions with 5-drug combinations with some levels of suppressive interactions (hidden and net), Related to Table 5. Terms in **bold** have a significant positive association with suppressive interactions.

Term	Coefficient	Confidence Interval		p-value	Odds Ratio	Probability
		0.50%	99.50%			
Cell Wall	**0.498**	**0.374**	**0.621**	**3.289E-25**	**1.645**	**62%**
Folic Acid Biosynthesis	**0.677**	**0.586**	**0.767**	**1.343E-82**	**1.967**	**66%**
DNA gyrase	**0.457**	**0.366**	**0.548**	**1.416E-38**	**1.579**	**61%**
Ribosome, 30S	-1.296	-1.454	-1.142	8.45E-102	0.274	21%
Ribosome, 50S	**0.585**	**0.462**	**0.709**	**2.201E-34**	**1.795**	**64%**
AIC: 17234		Bonferroni-corrected α: 0.01			Degrees of Freedom: 13602	

SI Table 9. Logistic regression of the pairwise main mechanism of actions with 5-drug combinations with some levels of suppressive interactions (hidden and net), Related to Table 6. Terms in **bold** have a significant positive association with suppressive interactions.

Term	Coefficient	Confidence Interval		p-value	Odds Ratio	Probability
		0.20%	99.80%			
Cell Wall+Folic Acid Biosynthesis	-1.609	-2.078	-1.165	2.14E-24	0.200	17%
Cell Wall+DNA gyrase	-0.984	-1.397	-0.580	3.45E-12	0.374	27%
Cell Wall+Ribosome, 30S	-0.983	-1.617	-0.369	5.34E-06	0.374	27%
Cell Wall+Ribosome, 50S	**3.715**	**2.907**	**4.585**	**1.67E-37**	**41.06**	**98%**
Folic Acid Biosynthesis+DNA gyrase	**0.431**	**0.207**	**0.654**	**2.47E-08**	**1.540**	**61%**
Folic Acid Biosynthesis+Ribosome, 30S	**1.932**	**1.329**	**2.576**	**3.81E-19**	**6.900**	**87%**
Folic Acid Biosynthesis+Ribosome, 50S	0.193	-0.220	0.603	0.174	1.213	55%
DNA gyrase+Ribosome, Ribosome, 30S	**1.300**	**0.748**	**1.889**	**4.53E-11**	**3.669**	**79%**
DNA gyrase+Ribosome, Ribosome, 50S	0.030	-0.373	0.429	0.827	1.031	51%
Ribosome, 30S+Ribosome, 50S	-3.349	-4.018	-2.712	1.04E-49	0.035	3%
Cell Wall+Cell Wall	**0.322**	**0.197**	**0.448**	**1.44E-13**	**1.379**	**58%**
Ribosome, 30S+Ribosome, 30S	**0.395**	**0.272**	**0.521**	**4.36E-20**	**1.485**	**60%**
Ribosome, 50S+Ribosome, 50S	**0.521**	**0.395**	**0.650**	**4.36E-32**	**1.683**	**63%**
AIC: 16981		Bonferroni-corrected α: 0.0039			Degrees of Freedom: 13602	

Transparent Methods

Experimental set-up of Tekin et al. (2018)

The data set examined was originally collected and published in Tekin et al. (2018). A pathogenic *E. coli* strain CFT073 was isolated from human clinical specimens and obtained from ATCC (700928). A culture of CFT073 was streak-purified on Luria Broth (LB) (10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl) agar and a single colony was selected to make individual aliquots of bacteria stored in 25% glycerol and frozen at -80°C. For each day of experiments, a new aliquot was used, which was thawed and diluted by a factor of 10^2 in LB and a culture was grown for approximately 4 hours at 37°C.

Eight different antibiotics that span a range of mechanisms of action was used (Table 1): Ampicillin (A9518), Cefoxitin Sodium Salt (C4786), Ciprofloxacin Hydrochloride (MP Biomedicals 199020), Doxycycline Hyclate (D9891), Erythromycin (E6376), Fusidic Acid Sodium Salt (F0881), Streptomycin (S6501), and Trimethoprim (T7883) (Table 1). All drugs were obtained from Sigma Aldrich unless otherwise noted. Each antibiotic was prepared in solution in 100% DMSO, except for streptomycin which was dissolved in 50% DMSO.

Dose-response curves were generated using GraphPad Prism 7 (http://www.graphpad.com/quickcalcs/Ecanything1/) to estimate IC10, IC5, and IC1 for each antibiotic, using 20-step 2-fold dilutions beginning at 0.1mM. For fusidic acid, the concentration used to begin the 2-fold dilutions was 1mM, since using 0.1mM to begin the dilutions resulted in the inability to determine an IC50 using Graphpad Prism 7. Three concentrations at the sub-inhibitory level were used so that growth still occurred but was slowed in comparison to no-growth bacteria (Table 1). Once usable concentrations were determined, source plates (one plate with one antibiotic and two plates with two antibiotics combined in DMSO) were made using 100% DMSO except in the case of streptomycin where 50% DMSO was used.

All possible 2-, 3-, 4-, and 5-drug combinations of the antibiotics listed in Table 1 at each of the three possible drug concentrations were tested. This resulted in 13,608 5-drug-dose combinations, 5,670 4-drug-dose combinations, 1,512 3-drug-dose combinations, 251 2-drug-dose combinations, and 24 single drug treatments. Each well was filled on each experimental plate to a total volume of 50µL. 25µL of LB was pinned with 250nL of antibiotics from the appropriate source plates and 25µL of the inoculum (a 10^{-4} dilution of the over day culture). Plates were incubated at 37°C and read at OD_{590} every 4hr for 16hr. Each combination had a minimum of three replicates.

Calculation of growth measurements

Growth measurements for each well were approximated from the maximum linear slope of the log transformed optical density (OD) readings that occurred over each time step (0hr to 4hr, 4hr to 8hr, 8hr to 12hr, and 12hr to 16hr) as a relative proxy to an exponential growth rate. These growth measurements were then normalized to the positive no-drug control wells to determine relative fitness values. Fitness values below 5% were considered to be lethal and fitness values that were +100% were set back to be 100%. These fitness values were then used to evaluate drug interactions based on the methods used in Tekin et al. (2018).

Measurement of interactions by Tekin et al. (2018)

To measure the deviation from additivity, known as “net interactions,” Bliss Independence methods (Bliss, 1939) were followed. The Bliss independence method is widely used to categorize interactions (Sühnel, 1998, Meletiadis et al., 2005, Yeh et al., 2006, Petraitis et al., 2009, Zhao et al., 2014, Baeder et al., 2016, Koch et al., 2016, Liu et al., 2018). Bliss independence assumes that at a set concentration of an antibiotic the relative effect is completely independent of each other. A deviation from this expectation results in either a synergistic interaction (positive deviation, Figure 1) or antagonistic interaction (negative deviation, Figure 1).

To measure net interactions, methods outlined in Beppler et al. (2016), Tekin et al. (2016), and Tekin et al. (2018) were used. This framework is used to examine 2-, 3-, 4-, and 5-drug combinations but can also be expanded to *N* number of drugs (Tekin et al., 2018). To find the net interaction, or the deviation from additivity for N drugs (DA_N) the fitness effects (w) contributed by each drug alone are removed from the overall fitness effect ($w_{D_1,D_2,D_3\ldots D_N}$) assuming Bliss independence (Equation 1). Note that "Deviation from Additivity" could more accurately be termed "Deviation from Independence" but because of prior usage of the term DA in the field of systems biology and microbiology, we continue to use this terminology here.

$$\textbf{Equation 1}: [DA_N]_{D_1,D_2,D_3\ldots D_N} = w_{D_1,D_2,D_3\ldots D_N} - w_{D_1}w_{D_2}w_{D_3}\ldots w_{D_N}$$

After the initial interaction value is determined, a rescaling process is used to better distinguish between interaction types (Tekin et al., 2016). For rescaling, when the DA is synergistic one rescales to the lethal case. This is because when measuring growth, it is not possible to be deader than dead. If the interaction was not synergistic then it was normalized to the minimum fitness of an individual drug within the deviation from additivity formulas. Equation 2 shows the example for a 3-drug combination.

$$\textbf{Equation 2}: DA_{rescaled} = \frac{[DA_N]_{D_1,D_2,D_3\ldots D_N}}{\left|min(w_{D_1},\ w_{D_2},\ w_{D_3},\ldots w_{D_N}) - w_{D_1}w_{D_2}w_{D_3}\ldots w_{D_N}\right|}$$

Emergent interactions were also examined. An emergent interaction is the interaction that is unique to either the three, four, or five drugs being present within a combination. For example, when considering all possible drug effects that can be occurring within a single 3-drug combination there are a total of seven effects. First, all three individual drugs have their own effect. These effects are accounted for when we are determining the deviation from additivity. Next, there are three pairwise interactions that can also interact with the individual drug effects of the third drug. And finally, there is the emergent effect, which is the interaction that is strictly because of the three drugs being in combination. Similar to the DA calculations the emergent calculations (*E3*) removes the effects of the single drugs but then also removes the effects of the pairwise interaction only leaving the effects uniquely due to the 3-drug combination (Equation 3). This can then be rewritten only in fitness effects. (Equation 4).

$$\textbf{Equation 3}: E3 = DA_{X,Y,Z} - w_X DA_{Y,Z} - w_Y DA_{X,Z} - w_Z DA_{X,Y}$$
$$\textbf{Equation 4}: E3 = w_{XYZ} - w_X w_{YZ} - w_Y w_{ZX} - w_Z w_{YZ} + 2w_X w_Y w_Z$$

The same principals can be expanded out to accommodate *N* number of drugs within a combination Tekin et al. (2018). These emergent interactions were then rescaled in a similar way as the DA values as described in Tekin et al. (2018).

Analysis of Prevalence and Patterns of Suppression and Hidden Suppression

The median DA_N of drug-dose replicate experiments was used to determine patterns of suppression in three, four, and five drug-dose combinations. A cutoff value of $DA_N \geq 1.3$ to classify combinations as net suppressive was used. This cutoff value is based on the framework used by Beppler et al. (2017), which only examined 2-drug and 3-drug combinations. All combinations, regardless of net interaction, were screened for hidden suppression.

Following this identification of net interactions, "paths" were generated for each of the drug-dose combinations. A "path" is a unique heterarchical grouping containing one representative of each of all the lower-order combinations within the highest-order combination. These paths facilitate comparisons of nested fitness values within *N*-order combinations, which are used to determine cases of suppression and hidden suppression. For instance, when evaluating possible hidden suppression in a 4 drug-dose combination, pairwise drug-dose combination values can only be compared to those of 3 drug-dose combinations that they are a part of, rather than those of all possible 3 drug-dose combinations (Figure 3A). Fitness values of all combinations and single drugs were included in these paths, resulting in six

paths for each 3-drug-dose combination, 24 paths for each 4-drug-dose combination (Figure 3A), and 120 paths for each 5-drug-dose combination (Figure 3B).

To identify the presence of hidden suppression, the fitness of the highest-order combination $\left(w_{D_1,D_2,D_3\ldots D_N}\right)$ was divided by the fitness of the lower-order combination with the smallest fitness $\left(\min\left(w_{D_1,D_2,D_3\ldots D_{N-1}} \ldots w_{D_1,D_2}\right)\right)$ (Equation 5).

Equation 5. $$Hidden\ suppresion \Leftrightarrow \frac{w_{D_1,D_2,D_3\ldots D_N}}{\min\left(w_{D_1,D_2,D_3\ldots D_{N-1}},\ldots,w_{D_1,D_2}\right)} \geq 1.3$$

A value greater than or equal to 1.3 indicates the presence of hidden suppression. Once the presence of hidden suppression was determined within a combination, each path was examined in-depth for all possible hidden suppression relationships. The net interaction, representative fitness values of inclusive combinations, and single drugs were compared and used to assess if the combination could be considered a special case, listed in SI Table 1.

Data for combinations with any suppressive interactions, net or hidden, was analyzed through the use of logistic regression in R using the 'glm' function. The variables were first changed to binary, with 1 indicating presence and 0 indicating the absence of drug or the main mechanism of action creating the initial sets of predictors. Because hidden suppressive interactions require at least three drugs to be present to be defined, this makes it necessary for the logistic regression model to not have an intercept term. This is because the case where all dummy variables are zero corresponds to no drug being present, in which case any suppressive interaction is not possible by definition. Single drugs and 2-drug combinations were evaluated separately for a clearer interpretation of the data and to ensure model identifiability without removing variables. Coefficients, confidence intervals, p-values, odds ratios, and the probability from the logistic regressions are available in Tables 3-6 and SI Tables 2-9.

Program Languages Used

The data analysis is performed in MATLAB version 2015a, Python version 3.7.0, and R 4.0.2. PRISM was used by Tekin et al. (2018) for their study but was not needed in the reanalysis performed by this study. Measurement of interactions and interaction type determination was performed in MATLAB. Generation of paths and the identification of hidden suppression and special cases were performed in Python. The determination of the growth measurements and logistic regressions were performed in R.

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Chapter 4: The evolution of resistance to synergistic multi-drug combinations is more complex than evolving resistance to each individual drug component.

Abstract

Multidrug antibiotic resistance is an urgent public health concern. Multiple strategies have been suggested to alleviate this problem, including the use of antibiotic combinations and cyclic therapies. Here we examine how resistance to the combinations affects the susceptibility to each individual component, and conversely, how resistance to the individual components of highly synergistic three-drug combinations can affect the efficacy and interaction of those combinations. To evaluate this, we evolved multiple strains of resistant *Staphylococcus epidermidis* in the lab. We show that evolving resistance to four highly synergistic combinations does not result in cross-resistance to all of its components, nor does prior resistance to one component of the combination guarantee survival when exposed to the combination. Our results have also identified a potential sequence among the combinations and individual drugs that result in continual susceptibility to the treatment option next in the sequence. This study highlights the importance of considering higher-order drug combinations in cyclic therapies and how antibiotic interactions can influence the evolutionary trajectory of bacterial populations.

Introduction

Antibiotic resistance is a problem facing humanity on a global scale (Ventola, 2015; Ahmad & Khan, 2019; Hernando-Amado et al., 2019; Coates et al., 2020; Maillard et al., 2020). Not only is drug resistance to a single drug a threat to public health, but even more concerning, multi-drug resistance in bacteria is reducing the number of viable treatment options. Some

estimates show that by 2050 there will no longer be any effective antibiotic options unless new drugs are developed or discovered (Jin et al., 2020). Multiple strategies have been suggested to mitigate this ever-growing problem including cycling through antibiotic treatments (Nichol et al., 2015) and the use of combinational drug therapy (e.g., to combine antibiotics or combine antibiotics with alternative therapies, such as phage therapy) (Vivas et al., 2019). However, the use of combinational drug therapy introduces new factors to consider. Here we examine how evolved resistant bacterial populations respond to drug combinations and how drug interactions shape evolutionary responses to additional antibiotic exposure.

Bacteria evolve antibiotic resistance primarily through one of two ways: acquired resistance through spontaneous mutation and acquired resistance through horizontal gene transfer (Blair et al., 2015). Typically, for a single antibiotic, the resistance level is evaluated at the minimum inhibitory concentration (MIC). This is the concentration of an antibiotic that allows for 0% to 10% growth, depending on the specific method used when evaluating growth (Garrod, 1935; Eagle & Musselman, 1948; Haight & Finland, 1952; Thomson & Sanders, 1994). However, when multiple antibiotics with multiple mechanisms are used in combination, there are added complexities and nonlinearities (Loewe, 1953; Beppler et al., 2016; Tekin et al., 2016; Beppler et al., 2017). Now, the cell is no longer facing one specific mechanism to evolve resistance to but rather multiple mechanisms.

No matter how resistance evolves initially, the resulting mutations can affect the outcomes of future antibiotic exposures (Munck et al., 2014; Nichol et al., 2015; Barbosa et al., 2017; Gomez et al., 2017; Nichol et al., 2019; Santos-Lopez et al., 2019). If the specific mutations from the initial adaptation confer a higher tolerance to a different antibiotic, then it has gained cross-resistance. Cross-resistance is when resistance initially evolved to antibiotic treatment A indirectly gains

resistance to antibiotic treatment B (Haight & Finland, 1952; Sanders, 2001; Obolski et al., 2015). Alternatively, if the specific mutations confer a lower tolerance to a different antibiotic treatment (treatment B), then it is termed collateral sensitivity (Obolski et al., 2015; Pál et al., 2015). Exploiting these relationships to find combinations of antibiotics to be used cyclically can be one strategy of addressing the antibiotic resistance problem (Kollef, 2001; Brown & Nathwani, 2005; Masterton, 2005; Nichol et al., 2015; Nichol et al., 2019; Kavanaugh et al., 2021). This is because one goal of the cycle is always to have at least one viable antibiotic treatment available thus locking the bacterial population in a constant state of susceptibility to current treatment options.

Another proposed method for addressing multidrug antibiotic resistance is through the use of antibiotic combinations simultaneously (Vivas et al., 2019). These antibiotic combinations can be categorized by three main types of interactions resulting from the combined effect: (1) A combination is considered additive if the combination yields the expected response of the combined effects based on the single drugs alone. (2) A synergistic combination yields a stronger response when compared to additivity (Yeh et al., 2009). Synergistic combinations have a stronger selection strength, making it more likely for resistance mutations to quickly sweep through a population (Orr, 2000; Pepin & Wichman, 2008) and can cause faster adaptation rates (Hegreness et al., 2008). (3) Finally, an antagonistic combination yields a weaker response when compared to additivity. Antagonistic combinations are avoided in the clinic as they require larger doses to have the same killing efficiency as synergistic combinations do.

Intriguingly, antagonistic combinations have been shown to have several advantages for slowing the evolution of resistant strains (Hegreness et al., 2008; Michel et al., 2008; Ventola, 2015; Maillard et al., 2020). Synergistic two-drug combinations have been shown to increase the likelihood of drug resistance to evolve via spontaneous mutants. Conversely, antagonistic

combinations show a decrease in the likelihood of resistance evolving (Michel et al., 2008). Additionally, Hegreness et al. (2008) showed that antagonistic two-drug combinations slowed the rate of resistance evolution when compared to the rate of resistance evolution to two-drug antagonistic combinations. These findings highlight how antibiotic interactions can be leveraged to slow the evolution of multidrug antibiotic combination resistance.

In multidrug combinations, many factors contribute to the overall fitness effect of the combination. The first factors to consider are the effects of each drug acting alone. Next, we must consider the effects of the additive interactions between the smaller sub-sets of antibiotics and the other single antibiotics (or combination of antibiotics) in the mix. Finally, we need to examine the highest order emergent effect—that is, the effect of the interaction between all drugs present in the combination. All these factors influence how a combination will ultimately affect a bacterial population.

Numerous studies have examined the evolutionary effects of cross-resistance and collateral sensitivity among single antibiotics in multiple species (Munck et al., 2014; Nichol et al., 2015; Barbosa et al., 2017; Gomez et al., 2017). The trajectory of resistance evolution can be influenced by using knowledge of collateral effects to create a specific sequence of antibiotics to steer it away from resistance evolution (Nichol et al., 2015). In addition, some mutations, such as those affecting the ribosome, have been identified that increase the evolution of multidrug resistance (Gomez et al., 2017). Yet other studies have questioned just how predictable collateral effects of resistance evolution are. These studies have found high amounts of stochasticity and variability of these collateral effects in replicate populations (Barbosa et al., 2017; Nichol et al., 2019). Studies have also expanded into evaluating how pairwise combinations align with cross-resistance and collateral sensitivity (Munck et al., 2014; Raymond, 2019). These studies found

that bacterial populations exposed to collaterally-sensitive antibiotic combinations are less likely to evolve resistance to the combination. Conversely, antibiotic pairs with similar or the same cellular/physiological targets that result in cross-resistance tend to increase the likelihood of evolution of resistance to the combination (Munck et al., 2014).

When antibiotics are used in combinations, they are typically used at concentrations near or above the MIC (Martin-Loeches et al., 2010; Paul et al., 2014; Kleine et al., 2017). It was previously thought that weakening the selection pressure by using lower antibiotic concentrations might curb the rapid evolution of resistance. However, for some antibiotic combinations, the opposite is true. Short-term higher doses are more effective at preventing resistance evolution compared to weaker doses (Bollenbach, 2015). When exposed to one antibiotic, at sub-inhibitory concentrations, an SOS response system is induced in bacteria. This SOS response allows for damaged DNA to be bypassed by the cell which in turn allows for mutation rates to increase (Chow et al., 2021). Thus, the effect of combinations could also be dependent on the dosages.

Our study focuses on *Staphylococcus epidermidis*, a gram-positive bacterium that colonizes skin and mucosa. *S. epidermidis* was previously considered an innocuous commensal microorganism on the human skin. However, it has recently become known as an opportunistic pathogen that results in nosocomial infections, particularly in indwelling medical devices such as catheters (Otto, 2009). We investigate the effects of antibiotic resistance to both the individual antibiotics by themselves and the combinations of antibiotics. We examine four highly synergistic three-drug combinations where all antibiotics are at sub-inhibitory concentrations (< 30% inhibition). These combinations are made up of piperacillin and tetracycline, with a third antibiotic of either: chloramphenicol, doxycycline, erythromycin, or neomycin. Specifically, we

address the following questions: (1) How does resistance to a three-drug combination affect the sensitivity to individual components of the combination? (2) Conversely, how does resistance to components of a three-drug combination affect sensitivity to the combination? (3) How does the interaction of a combination change after bacteria evolve resistance to one part of the antibiotic combination?

Materials and Methods

Creation and Isolation of Resistant Mutants

Eight strains of resistant *Staphylococcus epidermidis* (ATCC 14990) were independently evolved in a stepwise manner to each of the six antibiotics (Table 4-1) and each of the four drug combinations (Table 4-2). From here onward, individual antibiotics will be spelled out while combinations of antibiotics will be listed using their abbreviations (Table 4-1). For example, a combination consisting of piperacillin, tetracycline, and erythromycin will be listed as PIP+TET+ERY.

To start the initial populations, we prepared a highly dense cell culture of *S. epidermidis* by pinning the parental strain of *S. epidermidis* into 200μL per well of a 96-well plate of Lysogeny Broth (LB) media (10g tryptone, 5g yeast extract, and 10g NaCl) and incubated the culture for ~16 hours at 37°C shaking at 130rpm. To evolve resistance to a single antibiotic (those listed in Table 4-1), the highly-dense cell culture was then pinned over to a 96-well plate containing 200μL per well of LB and the antibiotic concentration for day 1, beginning with 50% of the parental minimum inhibitory concentration (MIC). The antibiotic concentration was continually doubled every 48hrs over 10 days, roughly 100 generations, resulting in a final drug concentration of 800% of the parental MIC. To evolve combination-resistant strains, we also

began with the parental strain of *S. epidermidis.* Similar to the creation of our single-drug-resistant mutants, we utilized a high-density cell culture of the parental strain. These high-density cell cultures were then pin-transferred into wells of a new 96-well plate filled with 200μL of LB media with one of the four, drug combinations, as listed in Table 4-2. These 96 well-plates were then incubated at 37°C for approximately 24 hours. Pinning the cell cultures into fresh media and antibiotics occurred every 24 hours and continued for 10 days or approximately 100 generations. Drug concentrations for all different drug combinations remained at constant levels for the entire time of the experimental evolution. This was done to keep the interactions within a combination constant (Berenbaum et al., 1983).

Once the 10-day experimental evolution component concluded, we isolated a single colony from each independently evolved population (each well). For the antibiotic chloramphenicol, resistant mutants were collected at 400% of the parental MIC, since growth would not occur at any higher concentrations. We isolated and confirmed resistance by streak purifying onto LB agar with 800% parental MIC of the respective antibiotic (400% parental MIC for chloramphenicol) or the respective antibiotic combination. We took the selected colonies from the streak purification and grew them in 2mL Luria Broth and incubated the culture for ~16 hours at 37°C shaking at 160rpm. This cell culture was then re-purified again on LB agar with the respective 800% (or 400% for CHL) parental MIC of the respective drug or the respective antibiotic combination. Master tubes with aliquots were made from a single colony selected from each of the second purifications that were cultured and stored at -80°C (in 25% glycerol).

Determination of the Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations (MICs) were determined using a 20-step, two-fold serial dilution of the antibiotics starting at 2000μg/mL. The layout for determining the MIC

of all bacterial strains is seen below in Figure 4-1A. Each well had a total working volume of 200μL, was inoculated with 50μL 1×10^5 CFU/mL, and incubated at 37°C for 22hr (Reller et al., 2009). A total of four technical replicates for each MIC determination was done. The data from all four replicates were pooled together into the 'drc' package in R to model the dose-response curve (Ritz et al., 2015; Ritz et al., 2016). This model was then used to estimate the concentration of antibiotics needed to inhibit growth by 95%. We also included negative controls on each of the 96 well-plates to confirm that there was no contamination of our media.

Combination Interaction Determination

The single-drug resistant mutants showed a phenotype that aggregates at the bottom of the standard flat-well plates. This is not uncommon when resistance evolves (Cushnie et al., 2007; Haaber et al., 2012; Dastgheyb et al., 2015; Ritz et al., 2015; Secor et al., 2018; Lozano-Huntelman et al., 2019). This aggregation does not allow for an accurate OD reading that correlates to the number of cells in the culture. To obtain accurate OD readings we used deep 96-well plates for the drug combinations to resuspend the cells in the media without creating bubbles. The plate set-up includes all high and lower-order combinations along with positive and negative controls as a means to compare the relative growth of bacteria (Figure 4-1B). The total working volume of these deep well plates was 400μL and each well was comprised of 100μL of LB and 100μL of the drug combination at their specified concentration, which is described in Table 4-2. Additionally, these deep-well plates were inoculated with 200μL 1×10^5 CFU/mL incubated for 24 hours at 37°C. After the 24-hour incubation period was complete, 200μL of the culture was transferred to a flat bottom 96-well plate to gather OD readings.

To calculate net and emergent interactions, we followed the Rescaled Bliss Independence (RBI) framework outlined in Beppler et al. (2016) and Tekin et al. (2016). There are other

approaches such as those that use Loewe Additiviity (Loewe, 1953), which unlike Bliss Independence, assumes that a drug cannot interact with it's self. However, the RBI framework was choses for it's unique ablity to determine the emergent *E3* value (descibed below) that quanitifies if a three-way interaction deviates from the combined effects of the three airwisse interactions within a three-drug combination (Beppler et al., 2016; Tekin et al., 2016). Briefly, in the RBI framework the net deviation from additivity, DA, is determined by only removing the fitness effects contributed by each drug alone (w_X, w_Y, w_Z) from the overall fitness ($\boldsymbol{w_{XYZ}}$) effect assuming Bliss independence (Equation 3) (Bliss, 1939). Once the net DA is determined the process can be done again, removing not only the additive contributions of each drug but also the effects of all lower-order interactions leaving only the emergent effect (Equation 4) (Beppler et al., 2016).

$$\textbf{Equation 3: DA} = \boldsymbol{w_{XYZ} - w_X w_Y w_Z}$$

$$\textbf{Equation 4: } \boldsymbol{E3 = w_{XYZ} - w_X w_{YZ} - w_Y w_{ZX} - w_Z w_{YZ} + 2 w_X w_Y w_Z}$$

After the initial interaction value is determined a rescaling process is used to better distinguish between interaction types (Tekin et al., 2016). The interaction values were rescaled following the same framework and methodology as used in Tekin et al. (2018).

Data Availability Statement

The raw data will be available through Mendeley Data once the manuscript has been accepted for publication.

Results

We cultivated eight independently evolved resistant strains of *S. epidermidis* for each individual antibiotic (tetracycline, piperacillin, chloramphenicol, doxycycline, erythromycin, or neomycin). We also created eight independently evolved resistant strains for each of the four

three-drug combinations: PIP+TET+CHL, PIP+TET+DOX, PIP+TET+ERY, and PIP+TET+NEO. These combinations are all highly synergistic and show at least 95% inhibition of the ancestral strain (ATCC 14990). We then assessed how the evolution of resistance can change both combination susceptibility and individual antibiotic sensitivity. We evaluated the minimum inhibitory concentration (MIC) at 95% inhibition. For the combination treatments, if relative growth (Equation 5) was at or below 5% the strain was considered to be susceptible to the combination. This definition of relative growth and susceptibility to a combination is consistent from this point forward. We also evaluated the change in interaction values when resistance evolved to a single antibiotic for each of the combinations.

$$\textbf{Equation 5: Relative growth} = \frac{\textbf{OD600 of treated poulation}}{\textbf{OD600 of a population in a no-drug enviorment}}$$

Resistance to combinations leads to changes in single antibiotic susceptibility

We first examined the collateral effects of how the evolution of resistance to the combinations can influence the MICs to all single antibiotics tested in this study. We performed a two-tailed, one sample T-test to evaluate the fold change in MIC $\left(\frac{MIC\ of\ resistant\ strain}{MIC\ of\ ansestrial\ strain}\right)$ by using $\mu = 1$. We then performed a Holm-Bonferroni correction to adjust for multiple comparisons. We found that resistance to any combination regimens, which contain a low concentration of piperacillin (inhibition of < 4%), led to potential cross-resistance to piperacillin alone. The average fold change of the MIC of piperacillin for each combination resistance set increased (Figure 4-2, Table 4-3). We also found that resistance to the combinations consistently led to collateral sensitivity to the tetracyclines (tetracycline and doxycycline). However, an exception to this trend was noted. The PIP+TET+NEO resistant strains did not display significantly different MIC between the ancestral strain to tetracycline (Figure 4-2, Table 4-3). For each of the remaining antibiotics, only two of the four combinations led to significant

changes in MIC. For instance, there was cross-resistance to chloramphenicol observed in the PIP+TET+CHL and PIP+TET+DOX resistant strains, but no significant changes in the chloramphenicol MIC when resistance evolved to PIP+TET+ERY and PIP+TET+NEO. Then, collateral sensitivity was observed in the PIP+TET+ERY and PIP+TET+NEO resistance strains to the erythromycin and the PIP+TET+DOX and PIP+TET+NEO resistant strains to neomycin. There was no significant change in the erythromycin MIC when resistance evolved to PIP+TET+CHL and PIP+TET+DOX, nor was there a significant change in the neomycin MIC when resistance evolved to PIP+TET+CHL and PIP+TET+ERY (Figure 4-2, Table 4-3).

Resistance to single drugs leads to changes in susceptibility to combinations

Evolving resistance to any single antibiotic typically resulted in an increase of resistance to the combinations to some degree (Figure 4-3, Table 4-4). We performed two-tailed, one sample T-tests to evaluate if resistance to a single drug resulted in more than 5% relative growth (using $\mu = 0.05$). We then used a Holm-Bonferroni correction to adjust for multiple comparisons. Piperacillin-resistant strains remained susceptible (not significantly greater than 5% relative growth) to all combinations having the lowers estimated growth means. The chloramphenicol-resistant strains were still susceptible to PIP+TET+DOX and were highly variable to PIP+TET+CHL, PIP+TET+ERY, and PIP+TET+NEO treatments. Tetracycline-resistant strains showed mixed susceptibility and high variability across all treatments. They remained susceptible to PIP+TET+CHL and PIP+TET+DOX but also showed decreased susceptibility to PIP+TET+ERY and PIP+TET+NEO. While doxycycline showed less variation and higher amounts of growth yet was not significantly higher than 5% relative growth. All other resistant strains (erythromycin, and neomycin resistant strains) led to the combinations no longer being effective. The erythromycin-resistant and the neomycin-resistant strains showed the overall

strongest resistance to the combinations with some individual strains growing better in the presence of the combination compared to the no drugs environment. The two tetracycline-resistant strains (tetracycline and doxycycline) showed high variability in growth across all treatments. Although the mean values for the tetracycline-resistant strains showed an increase in growth (over 5% relative growth) there was a large variation between each strain (Figure 4-3, Table 4-4).

Patterns of Cross Resistance and Collateral Sensitivity

We examined the patterns of the cross-resistant and collateral sensitivity networks for all the evolved strains (Figure 4-4A). These networks can change among specific replicate populations and even show contrasting outcomes when evolving resistance to the same antibiotic or combination. Thus, we focus our attention on the trends we see among most or all of our independently evolved biological replicates (Figure 4-4B). For instance, despite the synergistic interaction between the antibiotic combinations, the collateral effects varied depending on the specific antibiotic. The relationship between piperacillin, tetracycline, and all the combinations tested in this study, are consistent with a large majority of the independently evolved biological replicates (Figure 4-4B). Within this combination, there is a sequential order of treatments that consistently result in either cross resistance or collateral sensitivity depending on the order. If a bacterial population evolves resistance to a tetracycline, it will likely have cross resistance to any one of the highly synergistic combinations. The evolution of resistance to a combination leads to cross resistance of piperacillin. This sequential order could promote encompassing cross-resistance as a population evolves resistance to each step of the sequence. But if the order is reversed (evolving resistance to piperacillin first, then any one of the highly synergistic

combinations, and ending with evolving resistance to a tetracycline) the sequence shows collateral sensitivity instead.

Change in net and emergent interactions values

Drug interactions were calculated both on the ancestral strain and on the evolved resistant strains to examine the possible change in drug interactions in populations before and after evolving resistance. The interaction values of the combinations for the ancestral strain are listed in Table 4-2 and the degree of the change of the interaction values are shown in Figure 4-5.

We will first examine the net effects (DA), which are the overall effects that are due to all possible interactions within a combination. The net effects moved away from synergy and towards antagonism (the values became more positive) in cases that resulted in the three-drug combinations no longer being effective at largely inhibiting growth. Figure 4-5 shows just how frequently the net effects significantly changed. Piperacillin-resistant mutants were the only mutants to consistently have unchanged net effects (DA values). This means that the drug combinations remained highly synergistic ($p < 0.05$, two-tailed, one-sample T-test, $\mu = 0$).

In contrast to the ever-changing net effects *(DA)*, the emergent effects (E_3) were much more robust. The emergent effects are the effects of the interaction that is solely due to all three antibiotics being present in the combination. The emergent effects have fewer significant changes to the interaction value (E_3) (8 changes in the emergent effect versus 14 changes in net effect out of a total possible 24 opportunities to change) (Figure 4-5). The changes in the emergent effect varied between becoming more antagonistic (positive) or synergistic (negative) with no clear trend. The combination of PIP+TET+ERY was the most susceptible to changes in interaction values, both net and emergent, effects when antibiotic resistance evolves.

Discussion

We show that evolving resistance to a combination of antibiotics results in varying types of collateral effects to the individual antibiotics within the combination (Figure 4-2). This suggests resistance to a combination is not only the result of a stepwise accumulation of mutations. Initially, predictive models for the evolution of resistance to a multidrug combination assumed that the mechanisms of resistance are independent of each other (Komarova, 2006; Johnston et al., 2007; Saputra et al., 2018). That is, a fully-sensitive cell cannot become resistant to all drugs in the combination without undergoing multiple mutation events (Komarova, 2006). However, empirically it has been shown that these assumptions are not always supported with two-drug combinations (Gifford et al., 2019). Work has been done to try to incorporate these findings into predictive evolutionary models (Berríos-Caro et al., 2021). Furthermore, other factors such as tolerance—the ability for bacterial cells to survive but not actively grow in the presence of antibiotics—have also been shown to influence the predictions of these models (Liu et al., 2020). Even with the progress made and knowledge gained for two-drug combinations higher-order combinations (3+ antibiotics) are not as well studied which is why they are the focus of our study. This study provides further empirical evidence to support the idea that the evolution of resistance to a combination, including higher-order combinations, is not exclusively the result of a stepwise accumulation of resistance mutations to each individual antibiotic component within the combination.

Additionally, our results show that the evolution of resistance to a single component of a combination does not always make the combination ineffective (Figure 4-3). These findings suggest that it could be crucial to consider combinations of drugs in addition to individual drugs when searching for more viable antibiotic cycle discoveries. The interplay between the collateral

effects of sequential treatment and the interaction when used in combination of two antibiotics is currently a topic of interest. Combinations that are made up of pairs that have the potential for collateral sensitivities tended to slow the rate of antibiotic resistance adaptations (Barbosa et al., 2018). Antagonistic combinations have been shown to slow rates of adaptation and result in resistance profiles that are different from those only resistant to one component of the combination (Dean et al., 2020). While synergistic combinations can have higher efficacy they can also increase the selective advantage of resistance mutations over wild-type strains (Torella et al., 2010).

In this study, we incorporated all the components of the four three-drug combinations (piperacillin, tetracycline, chloramphenicol, doxycycline, erythromycin, neomycin) and the combinations themselves (PIP+TET+CHL, PIP+TET+DOX, PIP+TET+ERY, PIP+TET+NEO) to create a collateral effects network (Figure 4-4A). Using collateral effect networks (and even cellular hysteresis which is in a much more rapid timescale (Roemhild et al., 2018)) to develop sequential antibiotic treatments have been suggested as one approach to mitigate the problem of antibiotic resistance. In chronic infections (such as cystic fibrosis) these approaches are suggested to leverage phenotypes that after initial treatment and the following evolved resistance are still susceptible to other antibiotic options (Imamovic et al., 2018). However, the success of collateral effects is greatly determined on the pair of antibiotics involved. Sequential use of antibiotics that make up a synergistic pair have been correlated with increased cross resistance between the two drugs (Fuentes-Hernandez et al., 2015; Rodriguez de Evgrafov et al., 2015). However, using collateral sensitivities in principal may be leveraged in a clinical environment (Imamovic & Sommer, 2013). For example the use of sequentially treating *Escherichia* coli with collaterally sensitive drug pairs can have higher efficacy at lower does than using both antibiotics

simultaneously (Fuentes-Hernandez et al., 2015). In sequential treatments, collateral sensitives have even been shown to regenerate sensitivities to treatments that populations were previously resistant to (Dhawan et al., 2017; Barbosa et al., 2019). Antagonistic combinations have been shown to selects against resistance adaptations when the bacteria has already adapted to one of the antibiotics within the combinations (Chait et al., 2007). Our findings suggest a potential sequence of treatments where each step independently results in collateral sensitivities. This finding assumes that there is little effect from epistatic interactions among the accumulate adaptive mutations as population progresses through a sequence and that resistance adaptations give similar collateral effects. Epistasis has been a mechanism to aid in compensatory mutations to the fitness cost of multidrug resistance (Moura de Sousa et al., 2017; Das et al., 2020) and resistance mutations for the same antibiotic can lead to varying collateral effects (Maltas & Wood, 2019; Ardell & Kryazhimskiy, 2021). We encourage others to explore this in the potential sequence that may limit multidrug antibiotic resistance.

All of the combinations examined in this study use tetracycline and piperacillin and a third antibiotic allowing for a characterization of the interplay between piperacillin and tetracycline resistance evolution and resistance evolution to these four synergistic combinations. Depending on (1) the type of treatment (a combination or an individual antibiotic) a bacterial population evolved resistance to first and (2) the order of subsequent exposures, the collateral effects show opposing trends where one direction promotes collateral sensitivity and the other promotes cross-resistance (Figure 4-4B). For example, if a bacterial population evolves resistance to piperacillin, the collateral effect would be collateral sensitivity to any one of the combinations. The collateral effect of the evolution of resistance to a combination would also be collateral sensitivity, in this case to a tetracycline. This sequential order could promote continual

sensitivity as a population evolves resistance to each step of the sequence. However, if the order is reversed—that is, evolving resistance to tetracycline first, then any one of the highly synergistic combinations, and ending with evolving resistance to piperacillin—the collateral effects of the sequence would be cross resistance across each step.

Let us first examine the mechanism of resistance evolution to tetracycline and piperacillin individually. Tetracycline is part of the tetracycline class, a family of antibiotics that inhibit protein synthesis. This is done by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site (Chopra & Roberts, 2001). Evolving resistance to a tetracycline is a textbook example of the removal of the antibiotic through efflux pumps to keep intracellular concentrations low rendering the antibiotic ineffective (Speer et al., 1992). This strategy has been associated with multi-drug resistance across multiple classes due to the non-specific nature of the efflux pump (Webber & Piddock, 2003; Blanco et al., 2016). Our results suggest that evolving more general resistance mechanisms such as efflux pumps is enough to gain cross resistance to the highly synergistic combinations of this study: PIP+TET+CHL, PIP+TET+DOX, PIP+TET+ERY, PIP+TET+NEO (Figure 4-3).

On the other hand, the other antibiotic that is included in all combinations is piperacillin, a β-lactam, and is part of the penicillin class. The main mechanism of resistance for this class is through the increased production of penicillin-binding proteins (PBPs), proteins that are specific to β-lactam resistance (Dever & Dermody, 1991). Piperacillin specifically evokes a paradox where it primarily selects for one variant of PBPs (PBP2b) even though its most reactive target is a different PBP (PBP2x). Both of these PBPs are essential and involved in peptidoglycan assembly resulting in resistance and leading to a change in morphology (Philippe et al., 2015). This change in morphology may account for some of the variability observed in the combination

resistant strains, which are suspected of evolving piperacillin resistance (Figure 4-3), due to noise in optical density readings (Stevenson et al., 2016). Our results show that strains with evolved resistance to piperacillin remained susceptible to the drug combinations. This would suggest that the evolution of a more drug-specific resistance adaptation, such as the PBPs selected for piperacillin resistance is not enough to gain cross resistance to the synergistic combinations (Figure 4-4).

We show here that resistance to any of the antibiotic combinations in this study can result in cross-resistance to piperacillin and collateral sensitivity to tetracycline. Resistance to piperacillin requires a very specific type of resistance mechanism: the increased production of PBPs (Dever & Dermody, 1991). Because the strains resistant to the combinations also show cross-resistance to piperacillin, it may suggest that the evolution of PBPs are a result of evolving resistance to the combinations. We also show that the evolution of resistance to the combinations leads to collateral sensitivity to tetracycline. The main mechanism of tetracycline resistance is through the increase of efflux pumps (Speer et al., 1992). The collateral sensitivity to tetracycline of the combination resistant strains may indicate a decrease in the number of efflux pumps or decreased efficiency of the efflux pumps within the cell. It would be ideal for future studies to identify the exact mechanism of resistance to the combinations studied here. This can help determine if resistance to tetracycline is not required as part of the evolutionary path for evolving resistance to these synergistic combinations or if there is a currently unknown target of the combinations due to emergent interactions.

Historically, net interactions (DA) have been the focus of evolutionary biologists (Chen et al., 2008; Zimmer et al., 2016; Cokol et al., 2017; Katzir et al., 2019) and ecologists (Sokol-Hessner & Schmitz, 2002; McCoy et al., 2012; Sitvarin & Rypstra, 2014) because of the

difficulties in determining and describing higher-order emergent interactions accurately. Within the past 15 years, there has been substantial progress at determining and analyzing higher-order interactions (Yeh et al., 2009; Palmer et al., 2015; Beppler et al., 2016; Tekin et al., 2016; Zimmer et al., 2016; Beppler et al., 2017; Cokol et al., 2017; Tekin et al., 2018; Lozano-Huntelman et al., 2021). In both net and emergent interactions, the effects of an interaction are not primarily due to the chemical interactions between the compounds but rather due to how each individual component affects the physiology of the cell and how those effects interact (Bollenbach et al., 2009; Bollenbach, 2015; Mason et al., 2017). To add to the potential complexities of antibiotic interactions, interaction types may change when the concentration of antibiotic change even if they are kept in the same ratios (Berenbaum et al., 1983), a shift in specific dose combination can lead to different evolutionary outcomes (Gjini & Wood, 2021). Once the physiology of the cell is altered through evolved resistance adaptations, these interactions have the potential to change.

This study is the first to examine how both net and emergent interactions of drug combinations change in response to antibiotic resistance evolution. By testing the synergistic combinations to both the ancestral strain and the single drug resistant strains, our results show that net interactions are more prone to change when a population evolves and changes (i.e. adapted antibiotic resistance) (Figure 4-5). This makes the net interactions a more dynamic factor in response to the accumulation, loss, or change of physiological functions throughout the evolutionary history of a population. This, genetic background, creates variability that can make unraveling evolutionary trajectories difficult. In contrast, emergent interactions appear to be more robust to these physiological changes and adaptations. Understanding how these emergent interactions can affect the evolutionary trajectory of populations will be key to creating long-

term plans to assess antibiotic resistance in natural and clinical populations. This is because although populations will continue to evolve and adapt to new and changing environments (Fitzgerald, 2019; Santos-Lopez et al., 2019) the possible effects of emergent interactions may be more likely to stay in effect due to their observed higher robustness, as shown in Figure 4-5.

In conclusion, we show that: (1) Evolving resistance to a combination of antibiotics being used simultaneously does not always lead to cross-resistance to all of the components of that combination. (2) The evolution of resistance to one component of a combination does not always lead to cross-resistance to the combination. (3) The evolution of resistance to a single antibiotic affects the net interaction more often than the emergent interaction of a combination. Our findings suggest that it is important to consider antibiotic combinations in addition to individual antibiotics when measuring and examining cross-resistance and collateral sensitivity networks. Using those methods, we have identified a sequence-specific cycle that can promote both types of collateral effects. This cycle includes piperacillin, one of the three-drug combinations (PIP+TET+CHL, PIP+TET+DOX, PIP+TET+ERY, or PIP+TET+NEO), and tetracycline. Depending on the order of the evolved resistance, this sequence can either promote cross-resistance or collateral sensitivity. This framework—examining both collateral sensitivity and cross-resistance across whole networks of interactions at both the additive and emergent interaction levels—could allow researchers to uncover more viable sequential/cyclical treatment options that would extend the useful life span of antibiotics currently available.

Competing interests

The authors declare no competing interests.

Tables

Table 4-1. Antibiotic list and properties on the ancestral strain of *Staphylococcus epidermidis* (ATCC 14990).

Antibiotic (Abbreviation)	Class	Mechanism of Action	Concentration Used (μM)	Relative Fitness to No Drug Control	MIC (μM)
Chloramphenicol (CHL)	Chloramphenicol	Protein Synthesis, 50S	90	0.989	1352.621
Doxycycline (DOX)	Tetracycline	Protein Synthesis, 30S	0.6	0.780	257.522
Erythromycin (ERY)	Macrolide	Protein Synthesis, 50S	0.05	1.000	0.511
Neomycin (NEO)	Aminoglycoside	Protein Synthesis, 30S	0.35	0.803	13.280
Piperacillin (PIP)	Beta-lactam	β-Lactam, Cell wall	0.6	0.966	1.627
Tetracycline (TET)	Tetracycline	Protein Synthesis, 30S	20	0.713	205.137

Table 4-2. Drug concentrations of synergistic three-drug combinations with net interaction (*DA*) and emergent interaction (*E_3*) values based on the ancestral strain of *Staphylococcus epidermidis* (ATCC 14990).

Three-Drug Combination	Drugs in Combination (Abbreviation)	Single Drug Concentration (µM)	Fitness Effect of the Combination	*Net interaction (DA)*	*Emergent interaction (E_3)*
1	A) Piperacillin (PIP) B) Tetracycline (TET) C) Chloramphenicol (CHL)	0.6 20 90	0.004	-0.97	-0.09
2	A) Piperacillin (PIP) B) Tetracycline (TET) C) Doxycycline (DOX)	0.6 20 0.6	0	-1	1
3	A) Piperacillin (PIP) B) Tetracycline (TET) C) Erythromycin (ERY)	0.6 20 0.05	0	-1	0.98
4	A) Piperacillin (PIP) B) Tetracycline (TET) C) Neomycin (NEO)	0.6 20 0.35	0	-1	-0.08

Table 4-3. Comparison of minimum inhibitory concentrations (MIC) between ancestral strain and strains with evolved resistance to one of the highly synergistic combinations. Significant differences after correcting for multiple test (via Holm-Bonferroni) are shown in **bold**.

Strain	Drug Treatment	Estimate	95% CI		t-statistic	p-value	*n*
			2.50%	97.50%			
PIP+TET+CHL	CHL	1.614	1.184	2.044	3.374	0.012	7
PIP+TET+DOX	CHL	2.353	1.190	3.517	2.750	0.029	7
PIP+TET+ERY	CHL	1.486	0.914	2.057	2.010	0.084	7
PIP+TET+NEO	CHL	1.487	0.660	2.313	1.392	0.207	7
PIP+TET+CHL	**DOX**	**0.023**	**0.018**	**0.027**	**-548.846**	**0.000**	**7**
PIP+TET+DOX	**DOX**	**0.031**	**0.021**	**0.041**	**-226.640**	**0.000**	**7**
PIP+TET+ERY	**DOX**	**0.019**	**0.015**	**0.023**	**-556.348**	**0.000**	**7**
PIP+TET+NEO	**DOX**	**0.031**	**0.021**	**0.041**	**-233.468**	**0.000**	**7**
PIP+TET+CHL	ERY	1.376	0.261	2.492	0.798	0.451	7

PIP+TET+DOX	ERY	0.771	0.536	1.006	-2.308	0.054	7
PIP+TET+ERY	ERY	0.801	0.665	0.936	-3.482	0.010	7
PIP+TET+NEO	**ERY**	**0.645**	**0.541**	**0.749**	**-8.052**	**0.000**	**7**
PIP+TET+CHL	NEO	1.090	0.265	1.916	0.258	0.804	7
PIP+TET+DOX	NEO	0.549	0.285	0.813	-4.041	0.005	7
PIP+TET+ERY	NEO	1.143	0.653	1.633	0.689	0.513	7
PIP+TET+NEO	NEO	0.677	0.388	0.967	-2.637	0.034	7
PIP+TET+CHL	**PIP**	**8.000**	**5.248**	**10.752**	**6.014**	**0.001**	**7**
PIP+TET+DOX	PIP	26.964	1.377	52.550	2.399	0.048	7
PIP+TET+ERY	PIP	2.714	1.327	4.101	2.922	0.022	7
PIP+TET+NEO	PIP	5.419	2.875	7.962	4.108	0.005	7
PIP+TET+CHL	**TET**	**0.779**	**0.666**	**0.892**	**-4.614**	**0.002**	**7**
PIP+TET+DOX	TET	0.781	0.590	0.972	-2.708	0.030	7
PIP+TET+ERY	TET	0.791	0.607	0.974	-2.697	0.031	7
PIP+TET+NEO	TET	1.241	0.585	1.897	0.869	0.414	7

Table 4-4. Comparison of relative growth between ancestral strain and strains with evolved resistance to one of the single drug components. Significant differences after correcting for multiple test (via Holm-Bonferroni) are shown in **bold**.

Strain	**Combination Treatment**	**Estimate**	**95% CI**		**t-statistic**	**p-value**	***n***
			2.50%	**97.50%**			
CHL	PIP+TET+CHL	0.219	-0.246	0.685	0.860	0.418	7
DOX	PIP+TET+CHL	0.533	0.109	0.957	2.694	0.031	7
ERY	**PIP+TET+CHL**	**1.295**	**0.877**	**1.713**	**7.044**	**0.000**	**7**
NEO	**PIP+TET+CHL**	**1.157**	**0.686**	**1.627**	**5.565**	**0.001**	**7**
PIP	PIP+TET+CHL	0.045	-0.018	0.107	-0.195	0.851	7
TET	PIP+TET+CHL	0.433	-0.021	0.886	1.996	0.086	7
CHL	PIP+TET+DOX	0.048	0.006	0.090	-0.108	0.917	7
DOX	PIP+TET+DOX	0.459	0.104	0.814	2.726	0.030	7
ERY	**PIP+TET+DOX**	**1.226**	**1.002**	**1.451**	**12.844**	**0.000**	**6**
NEO	**PIP+TET+DOX**	**1.054**	**0.594**	**1.515**	**5.161**	**0.001**	**7**
PIP	PIP+TET+DOX	0.035	-0.007	0.078	-0.826	0.436	7
TET	PIP+TET+DOX	0.340	-0.027	0.708	1.931	0.102	6
CHL	PIP+TET+ERY	0.225	0.028	0.421	2.104	0.073	7
DOX	PIP+TET+ERY	0.458	0.201	0.715	3.759	0.007	7

ERY	**PIP+TET+ERY**	**1.054**	**0.781**	**1.328**	**8.694**	**0.000**	**7**
NEO	**PIP+TET+ERY**	**0.717**	**0.456**	**0.978**	**6.043**	**0.001**	**7**
PIP	PIP+TET+ERY	0.024	-0.009	0.057	-1.876	0.103	7
TET	PIP+TET+ERY	0.434	0.115	0.754	2.844	0.025	7
CHL	PIP+TET+NEO	0.152	0.003	0.301	1.623	0.149	7
DOX	PIP+TET+NEO	0.533	0.236	0.831	3.838	0.006	7
ERY	**PIP+TET+NEO**	**1.125**	**0.750**	**1.500**	**7.011**	**0.000**	**6**
NEO	PIP+TET+NEO	0.607	0.246	0.968	3.773	0.009	6
PIP	PIP+TET+NEO	0.036	-0.005	0.076	-0.824	0.437	7
TET	PIP+TET+NEO	0.351	0.130	0.573	3.213	0.015	7

Figures

A) B)

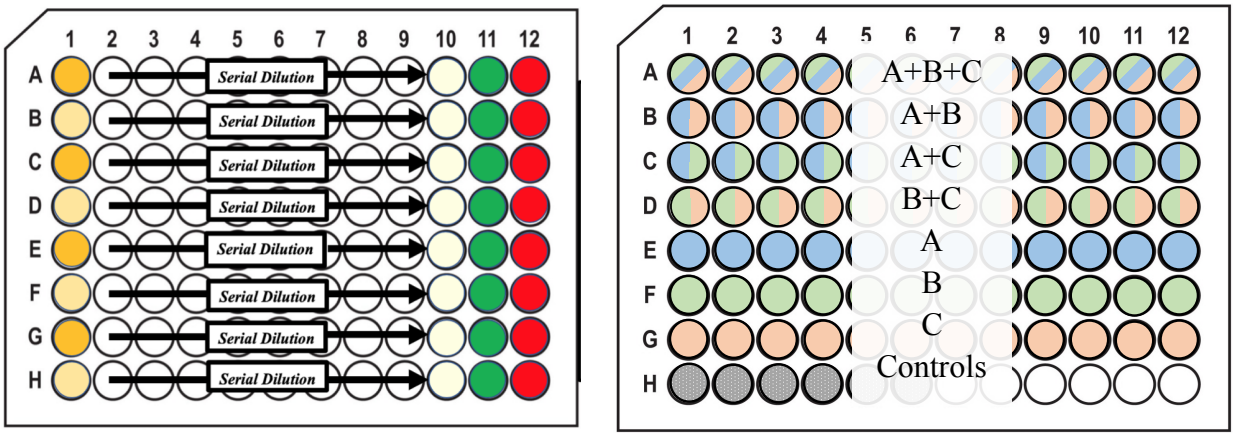

Figure 4-1. Plate Layouts for the MIC estimates and Deep Well-Plates. A) This figure shows each drug in concentration value in the 96 well-plates during the trials for evaluating the MIC of each bacterial strain. B) The diagram above illustrates an example of the layout used—a single deep well-plate—during the trials. This figure includes the locations for the high and lower-order drug combinations and their controls. These deep well-plates were incubated for 22hrs at 37℃ and later transferred onto a flat bottom 96 well-plate for OD reading.

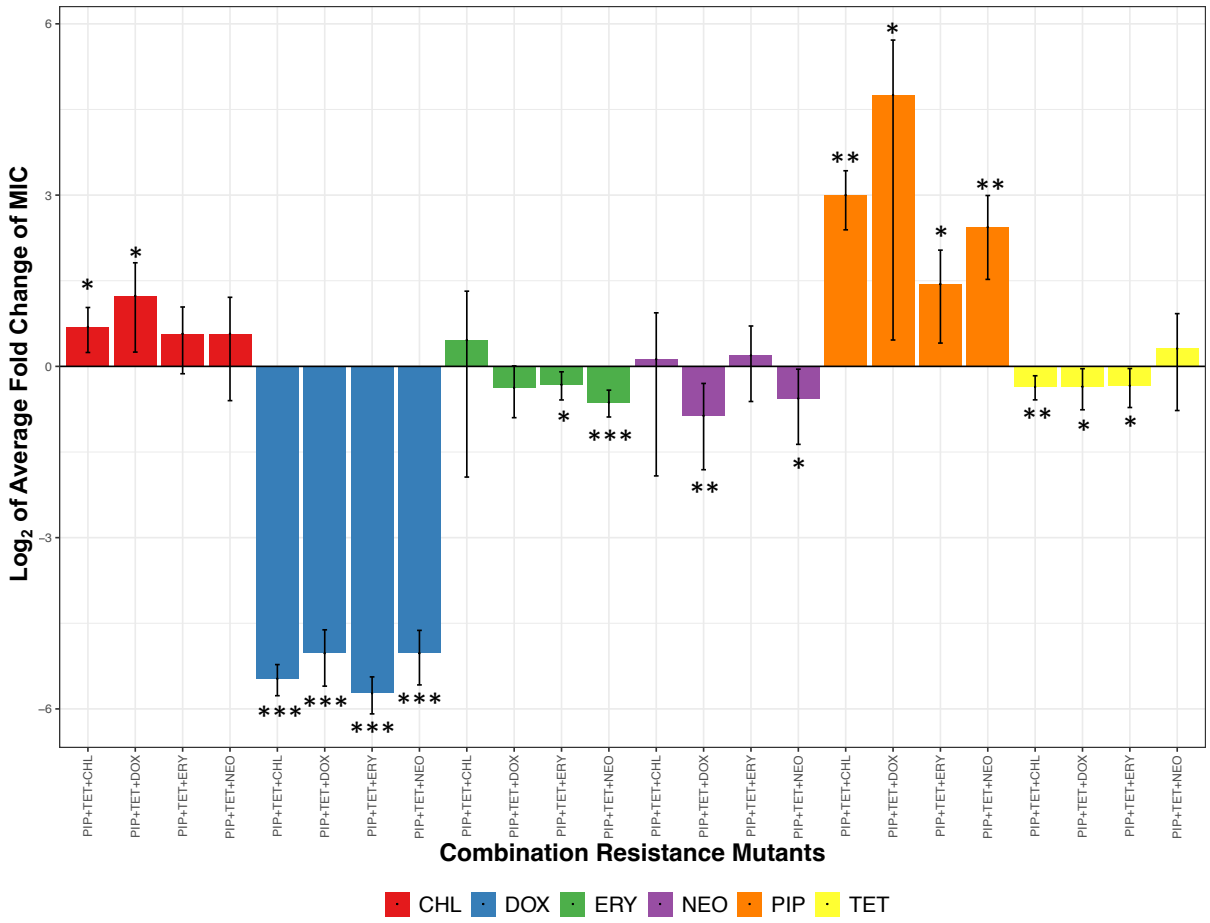

Figure 4-2. Resistance to synergistic drug combinations results in cross-resistance to piperacillin and collateral sensitivity to tetracyclines. The $\log_2$ of the average fold change in MIC after evolving resistance to a synergistic three-drug combination $\left(\frac{\text{MIC}_{resitant\ strain}}{\text{MIC}_{ansestrial\ strian}}\right)$ for each single drug component. The dashed line indicates the value of 1 where there is no change in the MIC of the resistant strains. A significant value below 0 indicates collateral sensitivity and a significant value above 0 indicates cross-resistance (two-tailed, one-sample T-test, $\mu = 1$). Error bars show 95% confidence intervals of the mean (n = 7). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

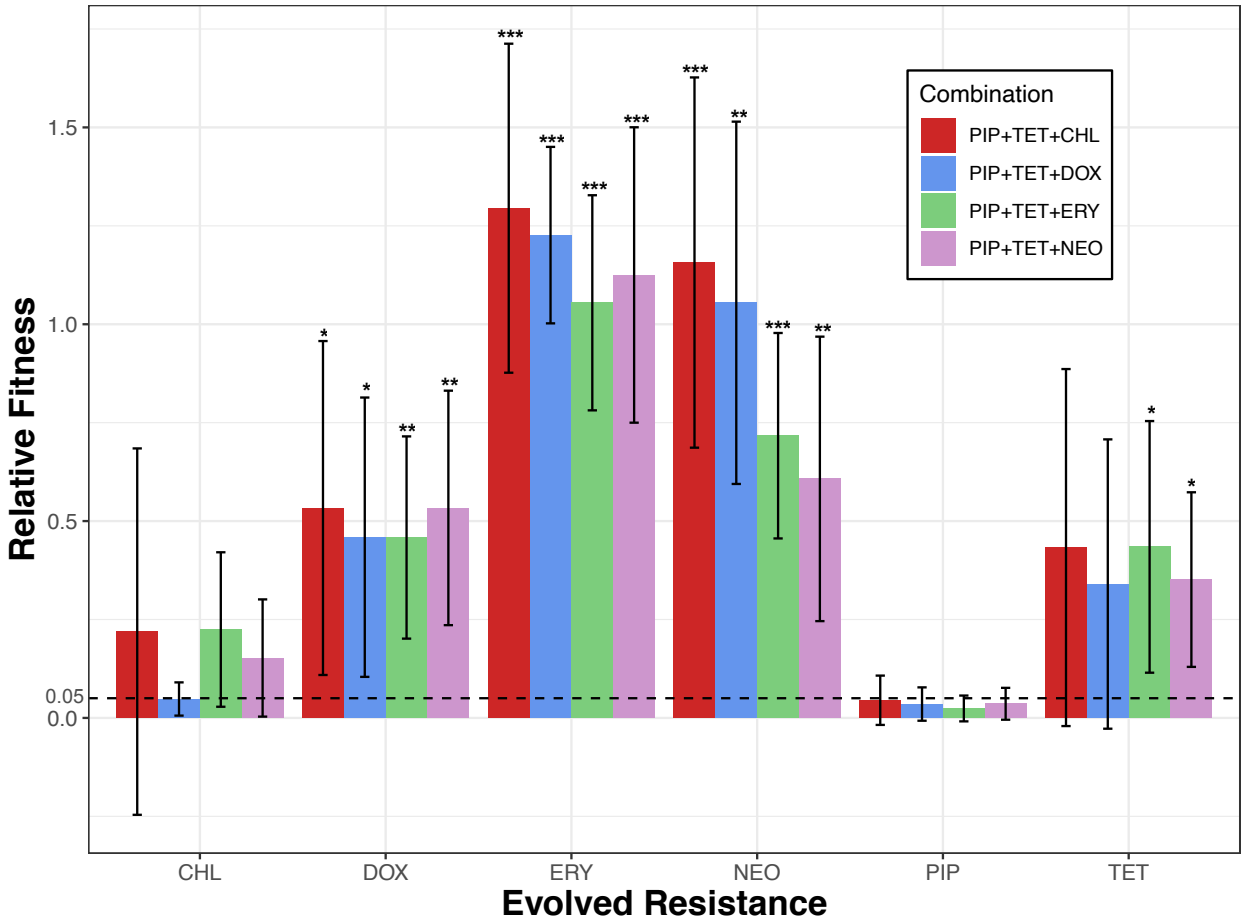

Figure 4-3. The evolution of resistance to some individual drug components results in a loss in susceptibility to the originally highly synergistic combination. The dashed line indicates a relative growth of 5%. Growth significantly higher than 5% gained some level of resistance to the combination, otherwise, there was no significant impact on the strain's susceptibility to the highly synergistic combination. Error bars show 95% confidence intervals of the mean (n = 7).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

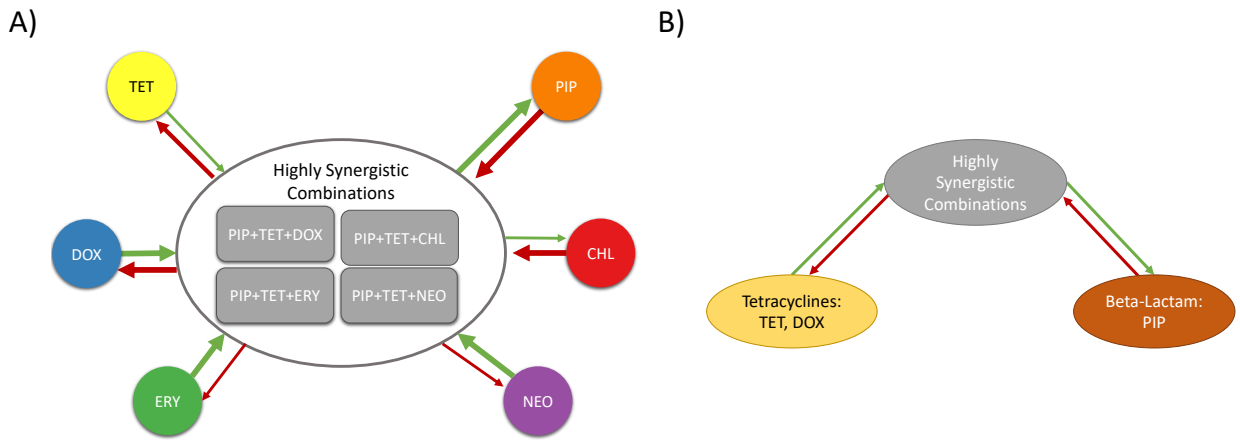

Figure 4-4. Patterns in the relationships between the four highly synergistic combinations and their individual components. Green arrows show a positive relationship for the bacteria: resistance to the combination/individual drug showed cross-resistance or loss of sensitivity to the individual drug/combination. Red arrows show a negative relationship for the bacteria: resistance to the combination/individual drug showed collateral sensitivity or remained completely susceptible to the individual drug/combination. A) Arrow weight shows how consistent the relationship is; the heavier the weight the more likely it is to observe the relationship. B) Possible viable antibiotic sequence, resulting in less resistance evolving.

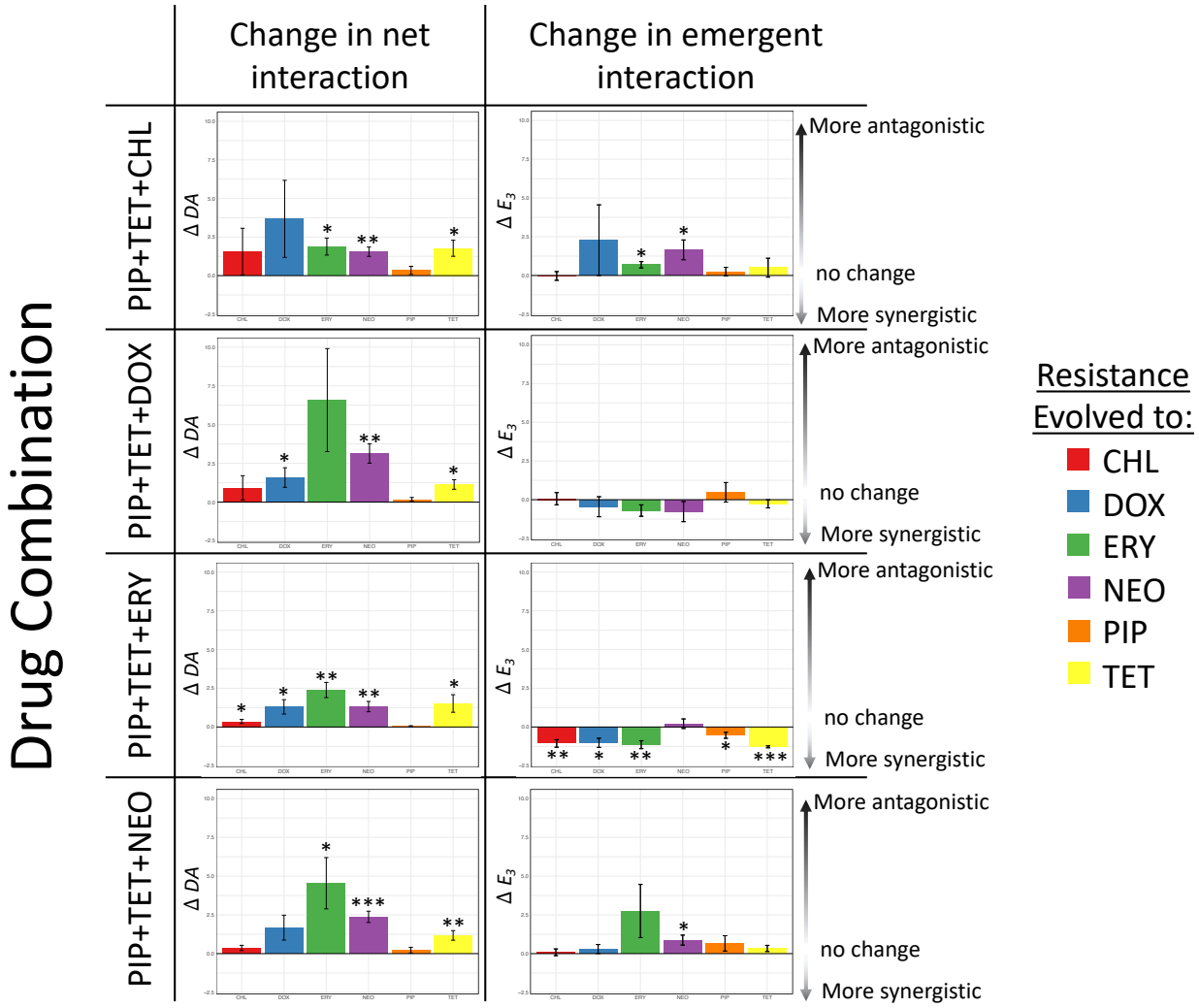

Figure 4-5. Net interactions are more likely than emergent interactions to be affected by the evolution of antibiotic resistance to a single antibiotic within the combination. Positive values indicate that the interaction is now more antagonistic with the evolved resistance than the ancestral strain. Negative values indicate that the interaction is now more synergistic with the evolved resistance than the ancestral strain (two-tailed, one-sample T-test, $\mu = 0$). Of the combinations tested, PIP+TET+ERY typically shows the most change in interaction values.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

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Chapter 5: Interactions within higher-order antibiotic combinations influence the rate of antibiotic resistance adaptation.

Abstract

The prevalence and strength of antibiotic-resistant bacteria have resulted in an arms race between the development of new treatment options and the evolution of resistance. Using multiple drugs in combination simultaneously has been suggested as a possible therapeutic solution to the antibiotic resistance problem. However, drug combinations introduce new factors to consider, including how the interactions among drugs influence the evolutionary process of adaptation to antibiotics. Antibiotic combinations are considered additive if the combined effect of the drugs is equivalent to the drugs acting independently. If a combination is more effective or less effective than the additive effect, then the combination is considered to be a synergistic or antagonistic interaction, respectively. We investigate how higher-order interactions—interactions involving three or more drugs—can affect the rate of evolution in *Staphylococcus epidermidis*. It has been previously established synergistic interactions between two drugs increase the rate of adaptation. However, it is unknown how higher-order interactions affect rates of adaptation. We examine the evolution of bacteria *S. epidermidis* in single drug, two-drug, and three-drug environments to determine how the interaction types of a combination may influence the rate and frequency of adaptation. We examine both the overall, or net, interaction of a combination as well as the emergent interaction—the interaction that is uniquely due to all drugs being present in a three-drug combination that is not due to pairwise interaction effects. We find that synergistic *net* interactions correlate with higher rates of adaptation, but that *emergent* interactions have no significant effect on the rates of adaptation.

Introduction

Antibiotic-resistant bacteria are present in the majority of environments worldwide, occurring in both clinical and non-clinical settings (Esiobu et al., 2002; Martinez, 2009; Wright, 2010). Through the overuse and misuse of antibiotics, bacterial populations evolve resistance (Ventola, 2015). This creates an arms race between the development of new antibiotics and the evolution of resistance resulting in a global health crisis (Levy & Marshall, 2004; Andersson, 2006; Naik et al., 2022). While more bacteria evolve single and multi-drug resistance (Bush et al., 2011; Spellberg & Gilbert, 2014), the discovery of new antibiotics has decreased dramatically (Nathan, 2004; Ventola, 2015). Antibiotic resistance has been projected to be responsible for 10 million deaths per year by 2050 if no new treatments or strategies are implemented (Tagliabue & Rappuoli, 2018). One solution to address this problem is to use antibiotics in combination (Fitzgerald et al., 2006; C. J. Brown et al., 2013; Foucquier & Guedj, 2015; E. D. Brown & Wright, 2016). However, combinations with multiple drugs introduce new factors to consider, including how the interactions between drugs influence the evolutionary process of antibiotic resistance.

When antibiotics are used in combination, the effects of the drugs themselves can interact with each other. These interactions can be categorized into three types: additive, synergistic, or antagonistic. An interaction is considered additive if the combination of drugs yields the same effect as if the single drugs were acting independently from each other (Bliss, 1939). A synergistic interaction gives a stronger response than expected; in the case of antibiotic interactions synergy would result in higher levels of growth inhibition (Bliss, 1939). *In vitro* studies have shown that these types of interactions can attain higher efficacy with lower concentrations however, they have been shown to promote the evolution of resistance (Hegreness et al., 2008; Michel et al., 2008). In contrast to synergistic interactions, antagonistic interactions yield a weaker response (less

inhibition of bacterial growth) than additive interactions (Bliss, 1939). Although higher concentrations of drugs within antagonistic combinations may be needed to obtain the desired degree of inhibition, they can also be more effective in preventing the evolution of resistance (Chait et al., 2007; Hegreness et al., 2008; Michel et al., 2008; Yeh et al., 2009).

The interactions of higher-order drug combinations (combinations that consist of three or more drugs) are more complex than the interaction of combinations consisting of only two drugs. This is because multiple interactions are occurring within a single higher-order combination. For example, in a three-drug combination, seven different factors attribute to the fitness effect of the combination. The first three factors are the effects of the three single antibiotics by themselves. The next three factors are the effects of the three pairwise interactions. The last factor is the effect of all three antibiotics interacting with each other. Thus, within a three-drug combination, there is a total of four interactions occurring simultaneously, the three pairwise interactions, and the interaction solely due to all three drugs being present (Beppler et al., 2016).

The interactions of a higher-order combination can be characterized in two ways: (1) the net interaction and (2) the emergent interaction. The net interaction of a higher-order combination is the overall effect of all possible interactions within the combination. The emergent interaction of a higher-order combination is the interaction that is due to all drugs being present and not a result of any lower-order interactions or single drug effects (Beppler et al., 2016). Most studies examine the overall fitness effects or *net* interactions of higher-ordered combinations (Zimmer et al., 2016; Katzir et al., 2019; Yilancioglu & Cokol, 2019); however, it is unknown if or how *emergent* interactions may influence the evolution of populations experiencing higher-order combinations of drugs. Emergent interactions have been found in multidrug higher-order (three or

more drugs) combinations (Beppler et al., 2016; Tekin et al., 2018). Understanding the properties of emergent interactions is crucial in having a complete picture of a complex system of stressors.

Here we examine how net and emergent interactions of three-drug combinations affect the rate of antibiotic resistance adaptation. The interactions of two-drug combinations have been examined extensively (Yeh et al., 2006; Yeh et al., 2009; K. Wood et al., 2012; K. B. Wood, 2016; Zimmer et al., 2016). It has been shown that they can influence the rates of resistance adaptations (Hegreness et al., 2008) and the likelihood of spontaneous resistance mutations (Michel et al., 2008). However, it is unclear how higher-order interactions affect the adaptation of resistance. We ask the following questions: 1) Does the net interaction of three-drug combinations affect the rate of adaptation? 2) Does the emergent interaction of three-drug combinations affect the rates of adaptation?

Materials and Methods

Bacterial strain and experimental evolution

We examined the evolution of *Staphylococcus epidermis* (ATCC 14990) populations to nine three-drug combinations (**Table 5-1**) all of the respective pairwise combinations (**Table 5-1**) and the single-drug treatments (Table 5-2). These drug combinations are comprised of a variety of antibiotics from different classes and have different main mechanisms of action (Table 5-2). For each drug treatment (three-drug combination, two-drug combination, and single drug) six populations were independently evolved. Each of these populations were evolved in one well on a 96-well plate with a working volume of 200 μL. For the first day of the experiment, plates were inoculated with cells via pin transferring (0.05 μL) of overnight cultures onto fresh plates. Plates were incubated at 37° C and had $O.D._{600nm}$ measurements taken every ten minutes for 23 hours

with a five-second orbital shake before each read. Populations were evolved over a fourteen-day period (roughly 150 generations). Every 24 hours, each population was pin-transferred (0.5 µL) over to a new plate containing fresh lysogeny broth media with the corresponding antibiotic treatment. Then, the new plate was incubated at 37° C and the $O.D._{600nm}$ measurements were taken every ten minutes for 23 hours with a five-second orbital shake before each read. The $O.D._{600nm}$ measurements taken on the first day of the experiment were used to determine the interaction values and fitness effects of the combinations. The $O.D._{600nm}$ measurements from all fourteen days were used to determine the rates of adaptation (see *Determination of Adaptation Rates* below).

Antibiotic Combinations and Interaction Values

The rescaled Bliss independence framework (RBI) (Tekin et al., 2016) was used to determine the interaction types and values of the combinations used here. For reference here is a brief overview of the framework. RBI uses Bliss independence (Bliss, 1939) as the additive model to evaluate interactions based on the relative fitness (w) to a no-drug control (Beppler et al., 2016; Tekin et al., 2016; Beppler et al., 2017; Tekin et al., 2017). Net interactions are determined using Equation 6. For example, in a two-drug combination, w_{AB} is the relative fitness of the bacterial population when treated with both drugs A and B in combination and $w_A w_B$ is the product of the relative fitnesses of being treated with drug A alone and drug B alone. If the deviation from additivity (DA) is a positive value it would indicate more growth than expected thus implying the interactions are antagonistic. A negative value would indicate a synergistic interaction.

$$\textbf{Equation 6: } \begin{cases} \textbf{two-drug combinations; } DA_{AB} = w_{AB} - w_A w_B \\ \textbf{three-drug combinations; } DA_{ABC} = w_{ABC} - w_A w_B w_C \end{cases}$$

Effectively the net interaction / deviation from additivity removes the additive effects of each individual drug. To find the emergent interactions we now must remove all the lower-order

interactions from the DA leaving the value of the highest order interaction possible. To do this we used Equation 7 which can all be expressed in terms of relative fitness resulting in Equation 8.

$$\textbf{Equation 7: } E3 = DA_{ABC} - DA_{AB}w_C - DA_{AC}w_B - DA_{BC}w_A$$

$$\textbf{Equation 8: } E3 = w_{ABC} - w_{AB}w_C - w_{AC}w_B - w_{BC}w_A + 2\,w_A w_B w_C$$

Then the net (*DA*) and emergent (*E3*) interactions were rescaled to enhance the ability to identify interactions occurring by normalizing to either the lethal cases (when evaluating synergistic interactions) or to the most effective single or a subset of drugs (when evaluating additive and antagonistic interactions). When rescaling non-synergistic emergent interactions, we normalized to relative effects from pairwise interactions. For more details on the rational and exact equations used please refer to Tekin et al. (2016).

Determination of Adaptation Rates

Rates of adaptation were determined by the change in the growth term (*GT*) (defined in Equation 9) over time following similar methods to Hegreness et al. (2008). The growth term used is a function of the growth rate (*r*) and the time it takes to grow to half of the carrying capacity (*K)*. We will refer to this as t_{mid}. This growth term was used to incorporate not only adaptations that increased growth rates but to also account for adaptations that reduced lag time promoting active growth in environments where antibiotics are subjugated to potential degradation (Li et al., 2016).

$$\textbf{Equation 9: } GT = \frac{r}{t_{mid}}$$

The growth rate (*r*), carrying capacity (*K)*, and t_{mid} were all determined by fitting Equation 10 to the OD data over time with the use of the Growthcurver (0.3.1) package in R (Sprouffske & Wagner, 2016).

$$\textbf{Equation 10: } \boldsymbol{OD(t) = \frac{K}{1+\left(\frac{K-N_0}{N_0}\right)e^{-rt}}}$$

The adaptation rate (α) is equal to the change between the initial and final growth term of a population (ΔGT) divided by the time to traverse a different fitness increase (t_{adapt}), Equation 11 (Hegreness et al., 2008).

$$\textbf{Equation 11: } \boldsymbol{\alpha = \frac{\Delta GT/2}{t_{\text{adapt}}}}$$

Results

Accounting for selection pressures

Before any comparisons between interactions and rates of adaptation were made, the effects a combination has on the fitness of a population and the rates of adaptation were determined. A Pearson correlation test was performed to measure the relationship between fitness and rates of adaption, showing a significant correlation (R = 0.23, p = 0.0017) (**Figure 5-1**). We then took the residuals of this correlation to determine if any type of interaction may influence the rate of adaptation. These residual values were used when comparing any subset of the data when looking for correlations (following similar approaches outlined in (Baltagi, 1998)) between rates of adaptation and interactions. All rates of adaptation have been corrected for this relationship.

Interactions and Adaptation Rates

To determine how the net interactions, correlate with rates of adaption, we performed a Pearson correlation on the populations that evolved and did not go extinct. We first observed the pooled data set comprising populations evolved to two-drug combinations and populations evolved to three-drug combinations. We observed a significant negative correlation (R= -0.23, p = 0.002)

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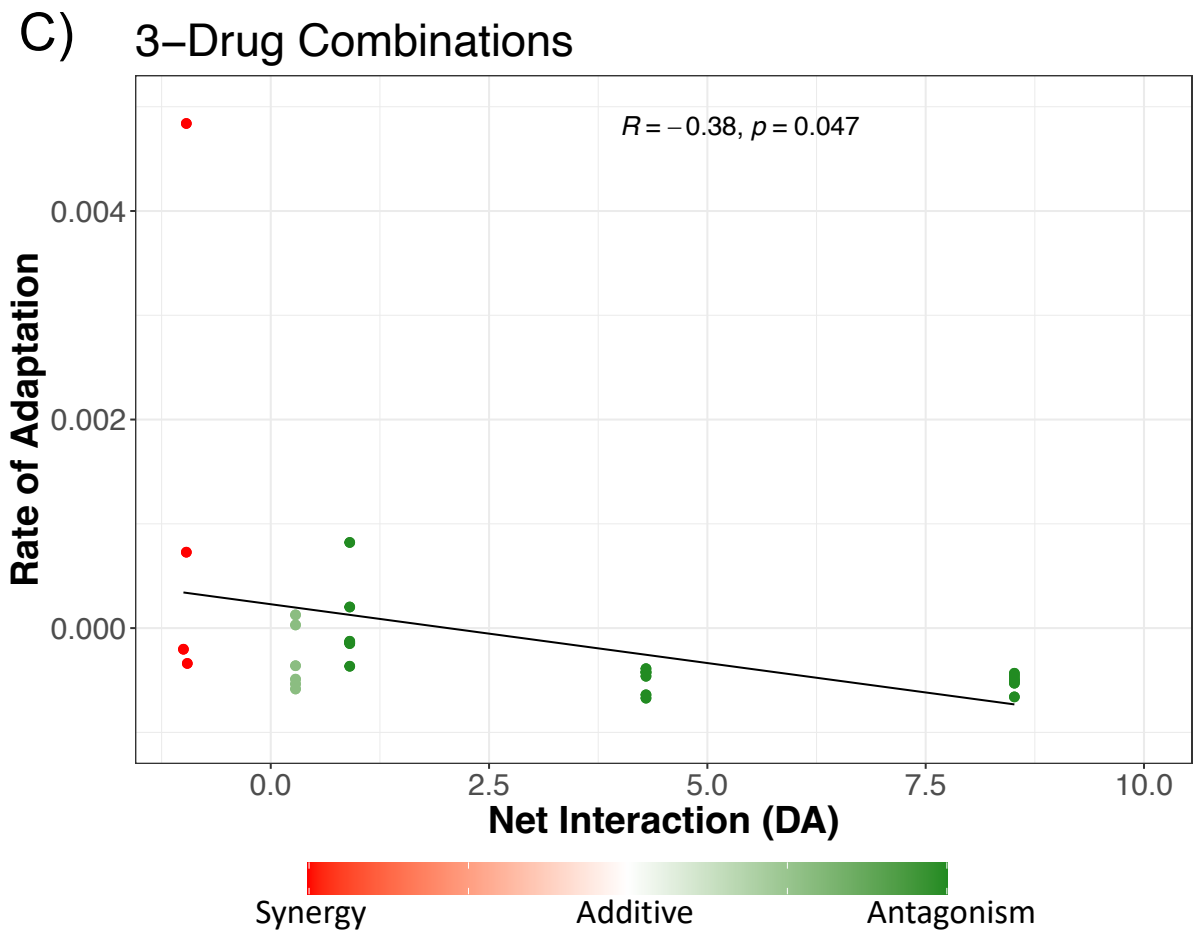


Figure **5-2**A). As the net interaction values decrease and become more negative (synergistic) the rate of adaptation increases. We then examined if this relationship remained with the two-drug and the three-drug combinations separately. We found that the significant negative correlation remained, but the degree of the correlation differed based on if there were two (R= -

0.17, p = 0.037) or three (R= -0.38, p = 0.047) drugs in the combination (

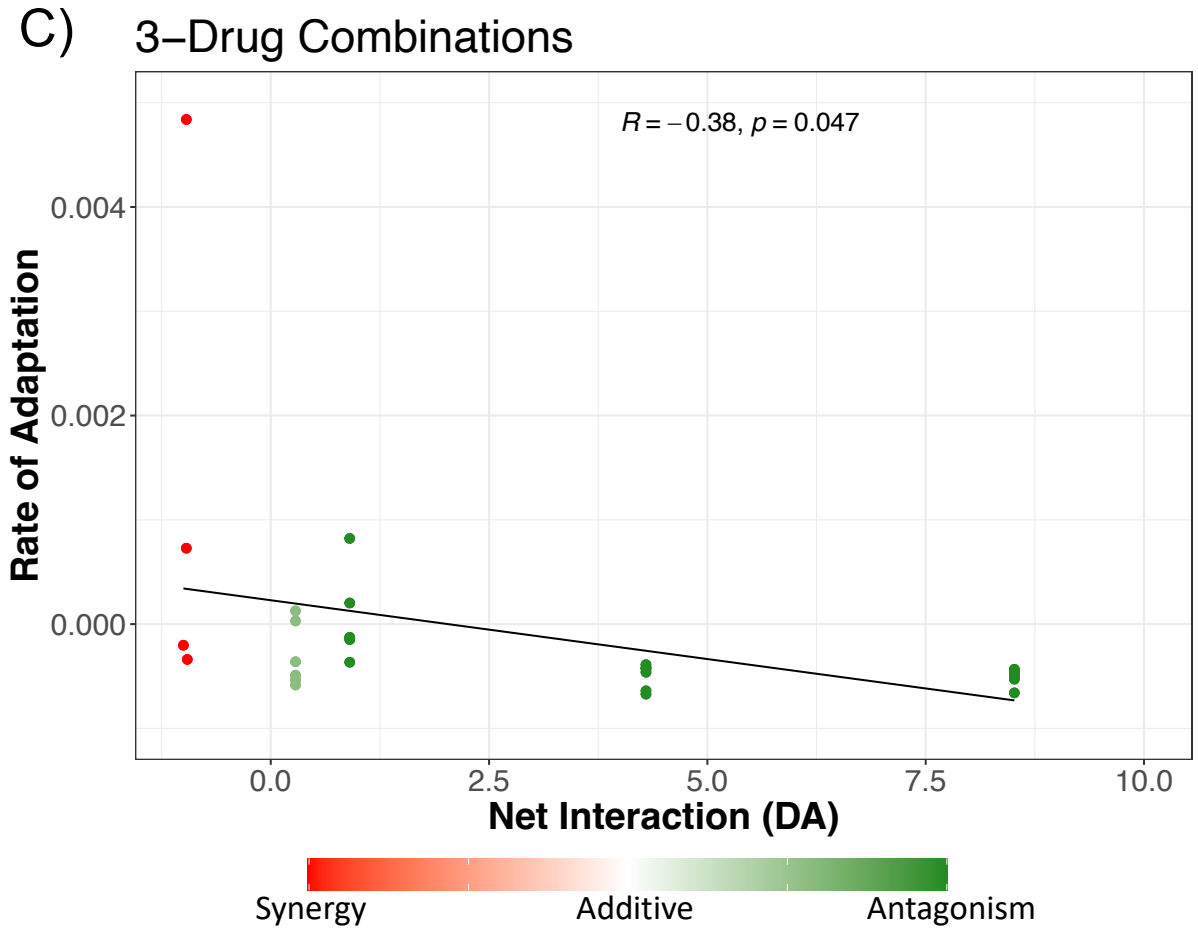


Figure **5-2**B and

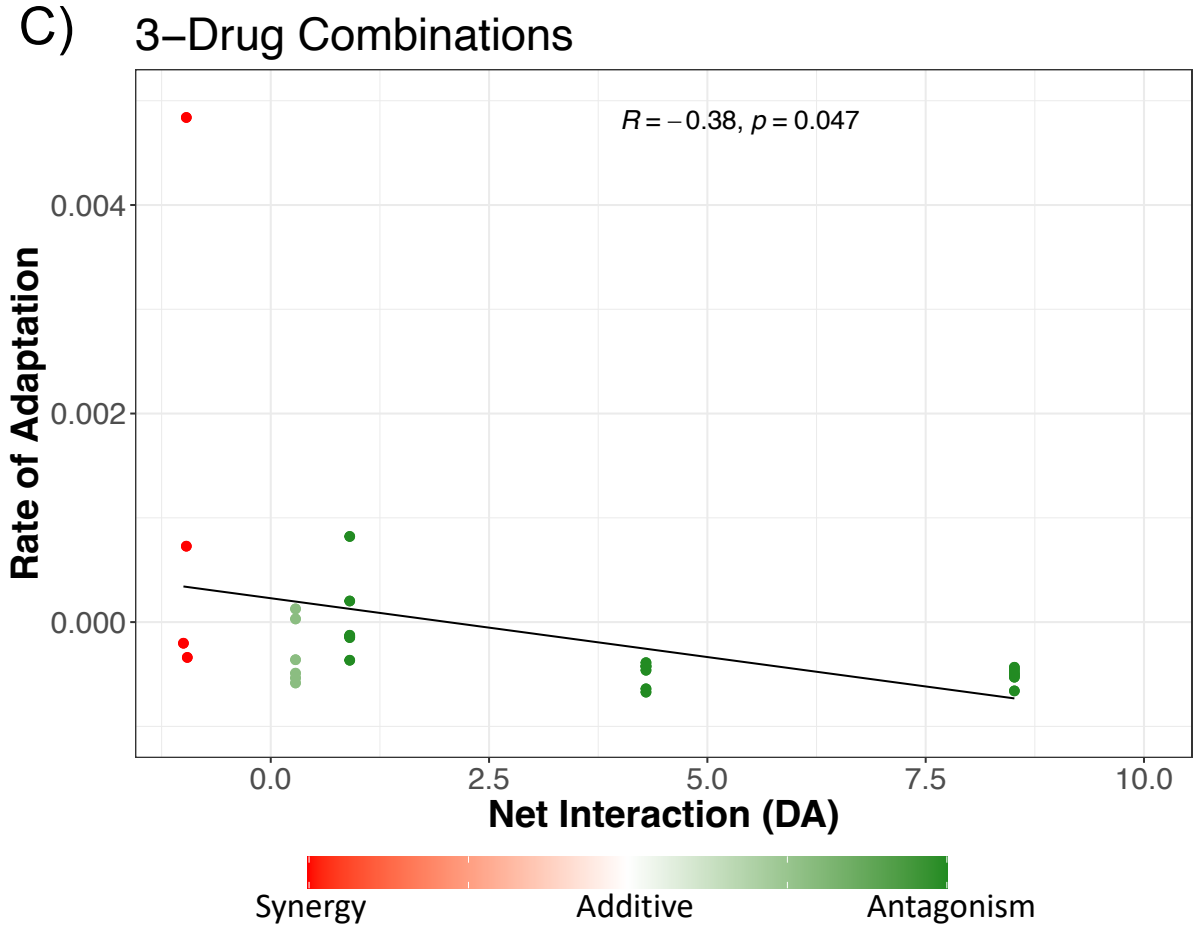


Figure **5-2**C respectively). We also asked if the emergent interactions of a three-drug combination correlate with rates of adaptation (**Figure 5-3**). We performed another Pearson correlation test and found no significant correlation (R= 0.1, p = 0.6).

Discussion

We asked how the interactions of a higher-order drug combination may correlate with the rates of adaption. We evolved multiple populations to a variety of three-drug combinations and all the corresponding two-drug and single-drug treatments over fourteen days. We found that the net interactions of both two-drug and three-drug combinations significantly correlate with the rates of

adaptation, where more synergistic interactions correlated with faster rates of adaptation (

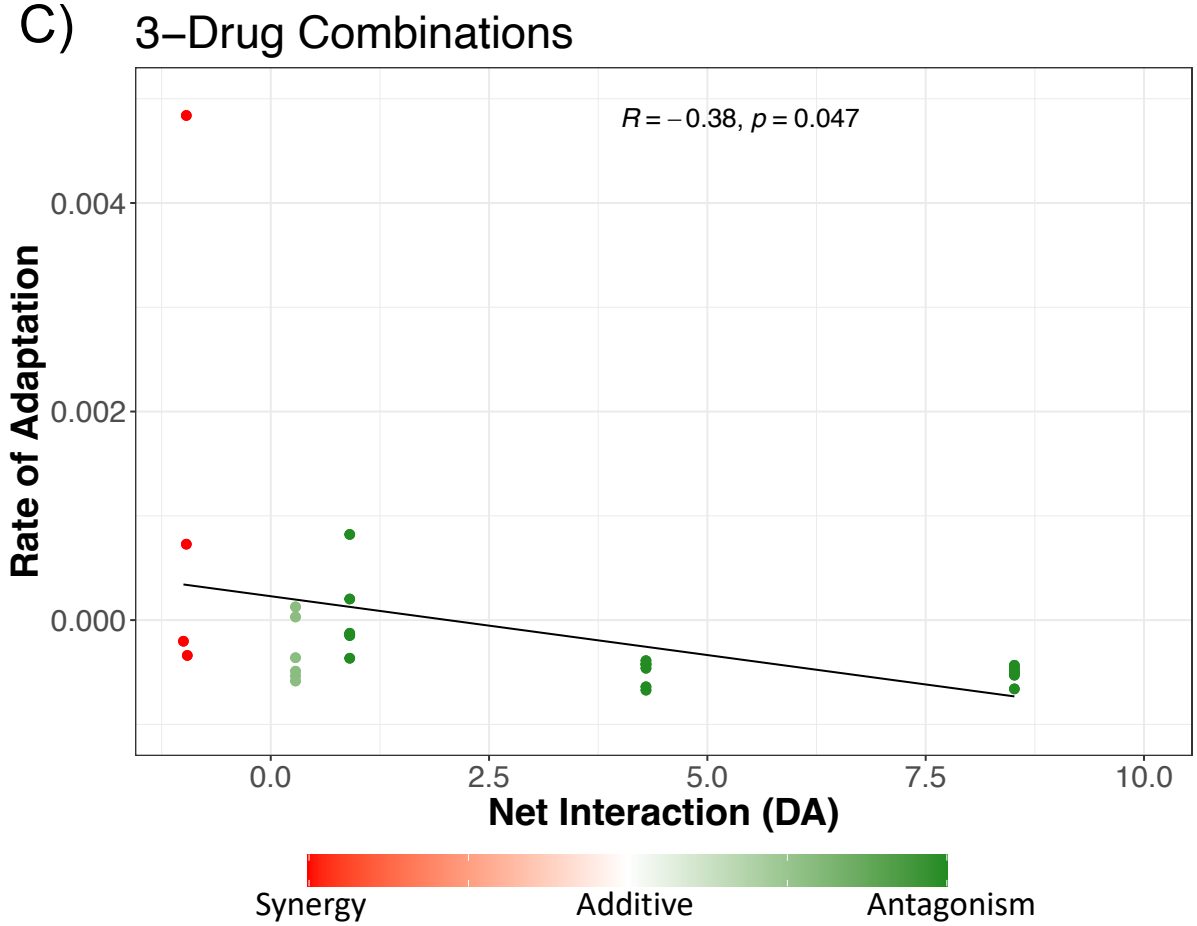


Figure **5-2**). We also determined that the emergent interaction of a three-drug combination did not correlate with rates of adaptation (**Figure 5-3**).

Hegreness et al. (2008) were the first to directly test if interactions between the antibiotics in a two-drug combination could correlate with adaptation rates. They evolved multiple populations of *Escherichia coli* to four different two-drug combinations for fifteen days (>150 generations). For each drug combination, a variety of doses and ratios were used and the interaction values were calculated for each drug-dose combination separately. This meant that two combinations with the same antibiotics but different doses could have different interaction values. Every 24 hours populations were transferred to fresh media that contained the same antibiotic combination at the same dosage. Growth was measured and growth rates were determined to

calculate adaptation rates. They found a significant positive correlation between the degree of synergy and rates of adaptation. That is, combinations with synergistic interactions correlated with higher rates of adaptation.

Multiple studies in two-drug combinations have suggested synergistic interactions promote antibiotic resistance evolution and antagonistic interactions limit antibiotic resistance evolution. Synergistic interactions have a higher likelihood of spontaneous resistance mutations (Michel et al., 2008). In addition, synergistic interactions have been shown to select for resistance while antagonistic interactions do not select for resistance (Chait et al., 2007). This is especially true when bacterial populations are faced with competition. It has been suggested that initial treatment with synergistic drug combinations could result in a higher bacterial load after treatment when compared to initial treatment with additive or antagonistic combinations (Pena-Miller et al., 2013). Antagonistic drug pairs maintain competitive interactions between the wild-type and single-drug resistant populations thus limiting the growth and further mutation of the single-drug resistant bacteria. Antagonistic combinations can maintain this competition because they are effective at killing off the entire wild-type bacteria (Torella et al., 2010).

Some previous studies did not find significant correlations between drug interaction and rates of adaptions or evolvability and rather suggest that collateral effects between a pair of drugs affect the evolution of resistance. Collateral effects are the unintentional changes in phenotypic response to other stressors because of previous adaptations. When evaluating collateral effects of antibiotic resistance, evolving resistance to one antibiotic may result in either increased resistance (cross-resistance) or increased sensitivity (collateral sensitivity) to another antibiotic (Haight & Finland, 1952; Sanders, 2001; Obolski et al., 2015). In two-drug combinations, mutations that confer drug resistance are typically not selected for if they also confer collateral sensitivity to the

other drug in the combination, limiting antibiotic resistance adaptation within a population (Munck et al., 2014). Pairwise drug combinations that are either cross-resistant or do not have collateral effects on each other have higher evolvability than those with collaterally sensitive drug pairs (Rodriguez de Evgrafov et al., 2015).

More recent studies have suggested alternative factors in addition to collateral effects that can influence rates of adaptation. Multiple populations of *Pseudomonas aeruginosa* were evolved to 38 different pairwise combinations (based on a range of drug interactions and collateral effects) to assess the antibiotic combination efficiency (ACE) (Barbosa et al., 2018). The ACE characterizes the ability of antibiotic combinations to limit bacteria survival and limit antibiotic resistance adaptation over time. Many of the drug combinations examined had synergistic interactions (24 interactions) but some had additive and antagonistic combinations (14 interactions each). By categorizing the ACE into two networks based on population extinction or adaption rates, they discovered that reduction in adaptation rates is driven by two factors: the adaptation to the component of a drug combination that has a stronger selection pressure alone and the specific collateral effect. There was no significant relationship between drug interactions and evolvability although they did observe instances that supported synergistic interactions selecting for resistance. In addition, synergistic combinations were the only combinations to experience extinctions despite having the same inhibitory levels as the other interaction types. Our current study supports these conclusions from Barbosa et al. (2018) regarding the importance antibiotic interactions can have on resistance evolution.

Another suggested factor that can influence rates of adaptation to a combination of antibiotics is the type of genetic response required for adapting resistance. The adapted genetic responses to single and two-drug combinations in *E. coli* were categorized to evaluate how

different genetic responses affect evolvability among different two-drug combinations (Jahn et al., 2021). The resistant mutation(s) selected by the drug combination was then compared to the mutation(s) selected for by each of the individual components alone. This resulted in four categories to describe the genetic response of adapting resistance to the combination. The four categories are as follows: 1) mutations conferring resistance to both drugs are the same and are selected by the combination; 2) mutations conferring resistance to both drugs individually are different and are selected by the combination; 3) mutations conferring resistance to both drugs individually are different but the combination only selects for one; or 4) mutations selected by combination are different than those selected by the individual drugs. Drug combinations that require novel mutations to gain resistance to the combination (category 4) limit the evolution of resistance compared to combinations where the mutations required for resistance are also selected by at least one of the components (categories 1, 2, and 3) (Jahn et al., 2021). When examining three-drug combinations the additional drug brings more complexities to evaluate by having to simultaneously consider three two-drug combinations. These additional complexities encountered when evaluating the genetic responses to a higher-order combination is similar to the additional complexities of determining the interactions of a higher-order combination. We hope that future studies will begin to examine the interplay between the genetic responses and the interactions of higher-order antibiotic combinations.

Most of the studies that did not conclude that interactions can influence rates of adaptation or evolvability evolved populations to a dynamic environment—that is the antibiotic concentrations of the combinations were increased as the populations evolved (Munck et al., 2014; Rodriguez de Evgrafov et al., 2015; Jahn et al., 2021). Even if the two antibiotics were kept with the same ratio the change of dosage can change the strength or even type of the interaction

(Berenbaum et al., 1983). This could mean that the populations being evolved may not have been adapting in response to the same interaction over the entire course of the experiments.

In contrast, Hegreness et al. (2008) used constant ratio and dosage of the drug combinations for the entirety of each evolution experiment. Hegreness et al. (2008) also tested a wide range of interactions from a single drug combination by choosing a variety of ratios and dosages. They found a correlation between interaction and rate of adaptation hold within the same combination of two drugs for all four drug combinations tested. Additionally, within the strongly synergistic combination tested (the combination of erythromycin and doxycycline) the highest rates of adaptation were found for dosages that have higher amounts of synergy. These rates are even higher than those of the populations evolving to the single drug components alone. In contrast, within the strongly antagonistic combination (ciprofloxacin and doxycycline) there was a decreased rate of adaptation compared to the single drug components alone. Our current findings further support the conclusion that net synergies increase the rate of adaptation to antibiotics.

But beyond that, this study is the first to directly test the relevance of emergent interactions regarding the evolution of a population. Studying emergent interactions has been historically difficult to do because it requires a large-scale data set with a full factorial design, where all possible subsets and individual factors are tested independently. But more recently, emergent interactions have been systematically measured and shown to be very frequent among antibiotic combinations (Tekin et al., 2018; Lozano-Huntelman et al., 2020) and ecological stressors (Diamant et al., 2022). Future studies can help elucidate the role emergent properties play in the evolution of a suite of population traits.

Tables and Figures:

Table 5-1. The combinations and concentrations of antibiotics used in three-drug combinations. Fitness is expressed by relative fitness to a no-drug control.

Three-Drug Antibiotic Combination	Net Interactions (DA)	Emergent Interactions (E3)	Fitness	Two-Drug Antibiotic Combination	Net Interactions (DA)	Fitness
CLI+FUS+TMP	10+	-0.545	2.069	CLI- FUS	-0.062	0.981
				CLI- TMP	5.503	2.987
				TMP- FUS	1.233	0.565
NEO+PIP+TMP	-0.967	0.394	0.049	NEO-PIP	1.069	1.277
				NEO-TMP	-0.140	1.017
				TMP-PIP	-0.890	0.162
GEN+PIP+TMP	-0.956	1.076	0.085	GEN-PIP	6.026	1.542
				GEN-TMP	-1	0
				TMP-PIP	-1	0
CHL+GEN+TET	8.516	0.185	2.139	CHL-GEN	-0.116	0.667
				CHL-TET	6.706	1.569
				TET-GEN	4.008	1.788
FUS+NAL+TMP	0.904	-1	1.110	FUS-NAL	-0.082	1.457
				FUS-TMP	2.656	1.310
				TMP-NAL	3.063	1.187
CHL+DOX+NAL	0.284	-0.614	1.814	CHL-DOX	2.823	1.431
				CHL-NAL	1.754	1.589
				NAL-DOX	1.222	1.869
CHL+ERY+NAL	10+	10+	0.239	CHL-ERY	-0.045	1.499
				CHL-NAL	1	0
				NAL-ERY	1	0
FUS+OX+TET	-1	1	0.010	FUS-OX	-0.223	1.196
				FUS-TET	-1	0
				TET-OX	-1	0
FOX+GEN+TET	4.297	-0.133	1.987	FOX-GEN	-0.024	0.920
				FOX-TET	2.771	1.417
				TET-GEN	5.271	2.089

Table 5-2. The class and the main mechanism of action for the 12 antibiotics used in this study. Fitness is expressed by relative fitness to a no-drug control.

Antibiotic	Abbr.	Mechanism of Action	Class	Concentration (μMol)	Fitness
Cefoxitin sodium salt	FOX	Protein synthesis, 50S	Beta-lactam; Cephalosporins	0.7	0.948
Chloramphenicol	CHL	Protein synthesis, 50S	Broad-spectrum	90	1.054
Clindamycin hydrochloride	CLI	Protein synthesis, 50S	Macrolides	0.01	1.023
Doxycycline hyclate	DOX	Protein synthesis, 30S	Tetracyclines	0.6	1.215
Fusidic acid	FUS	Protein synthesis, 50S	Fusidane	0.005	1.043
Gentamicin sulfate	GEN	Protein synthesis, 50S	Aminoglycosides	0.15	0.965
Nalidixic acid sodium salt	NAL	DNA Gyrase	Quinolone	20	1.492
Neomycin	NEO	Protein synthesis, 50S	Aminoglycosides	0.35	0.893
Oxacillin sodium salt	OX	Cell Wall	Beta-lactam; Penicillin	0.005	1.193
Piperacillin sodium salt	PIP	Cell Wall	Beta-lactam: Penicillin	0.6	1.061
Tetracycline	TET	Protein synthesis, 30S	Tetracyclines	20	0.810
Erythromycin	ERY	Protein synthesis, 50S	Macrolides	0.05	1.070
Trimethoprim	TMP	Folic Acid	Antifolate	1.5	0.065

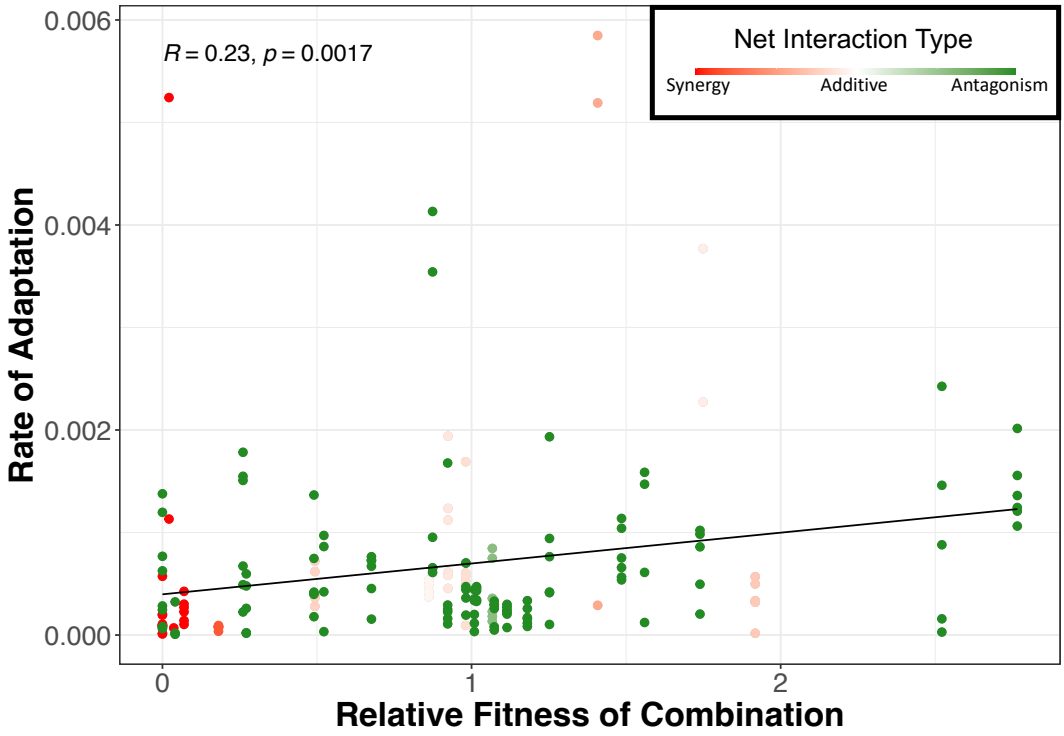
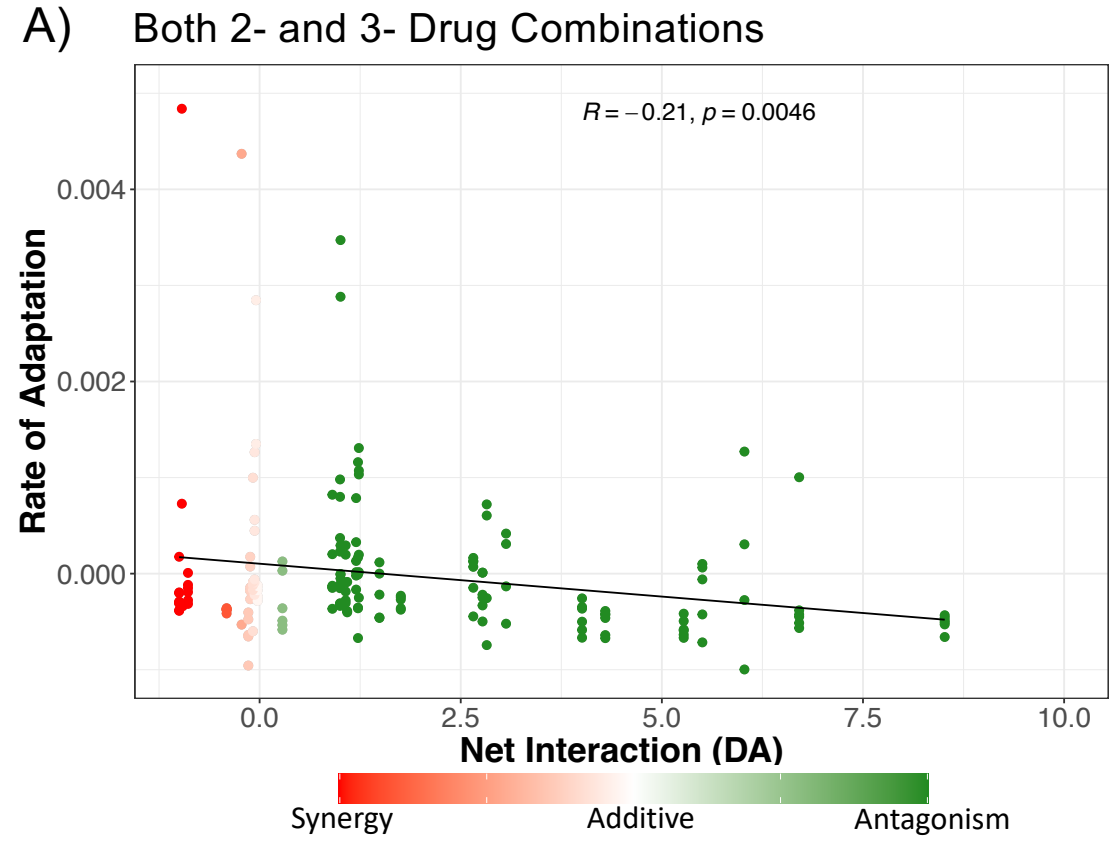
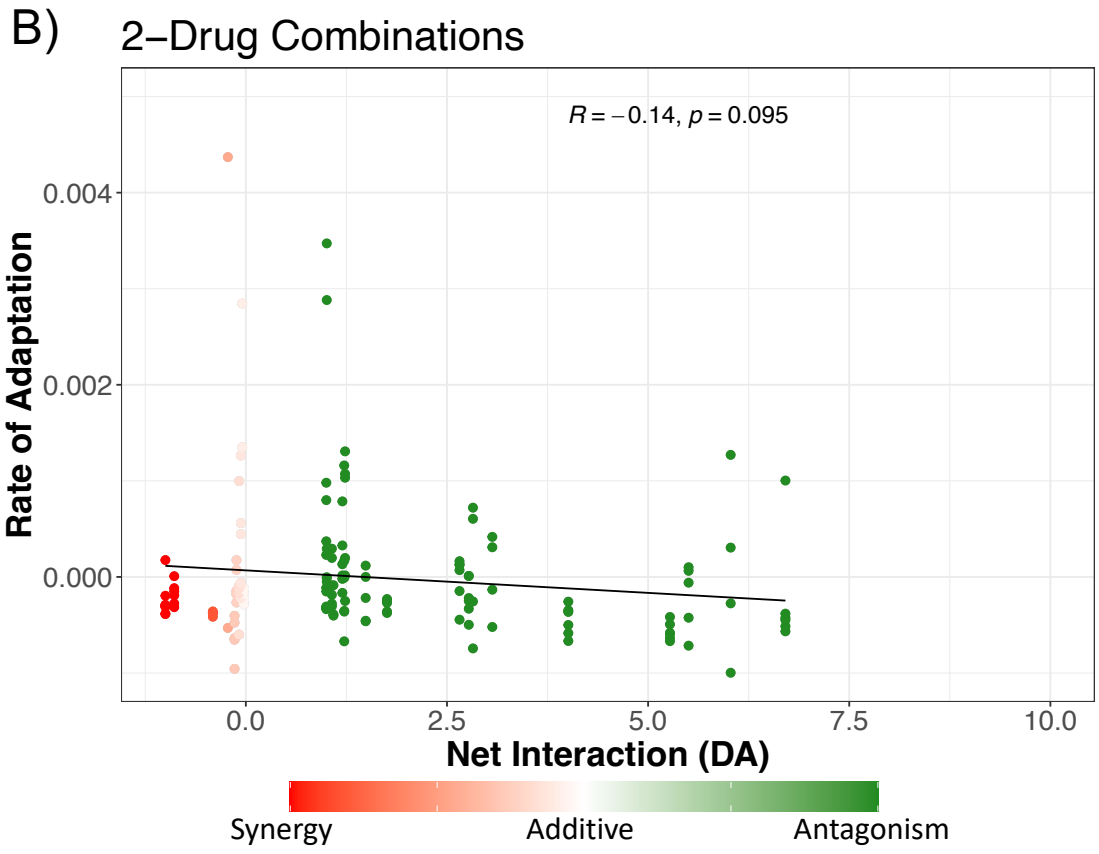


Figure 5-1. The correlation between relative fitness of the ancestral strain exposed to a combination and rate of adaptation. A Pearson correlation test was performed to measure the relationship between fitness and rates of adaption, showing a significant correlation (R = 0.23, p = 0.0017). The data from both two- and three- drug combinations were pooled together.


A)
Both 2- and 3- Drug Combinations
R = −0.21, p = 0.0046
Rate of Adaptation
0.004
0.002
0.000
0.0
2.5
5.0
7.5
10.0
Net Interaction (DA)
Synergy
Additive
Antagonism


B)
2–Drug Combinations
R = −0.14, p = 0.095
Rate of Adaptation
0.004
0.002
0.000
0.0
2.5
5.0
7.5
10.0
Net Interaction (DA)
Synergy
Additive
Antagonism

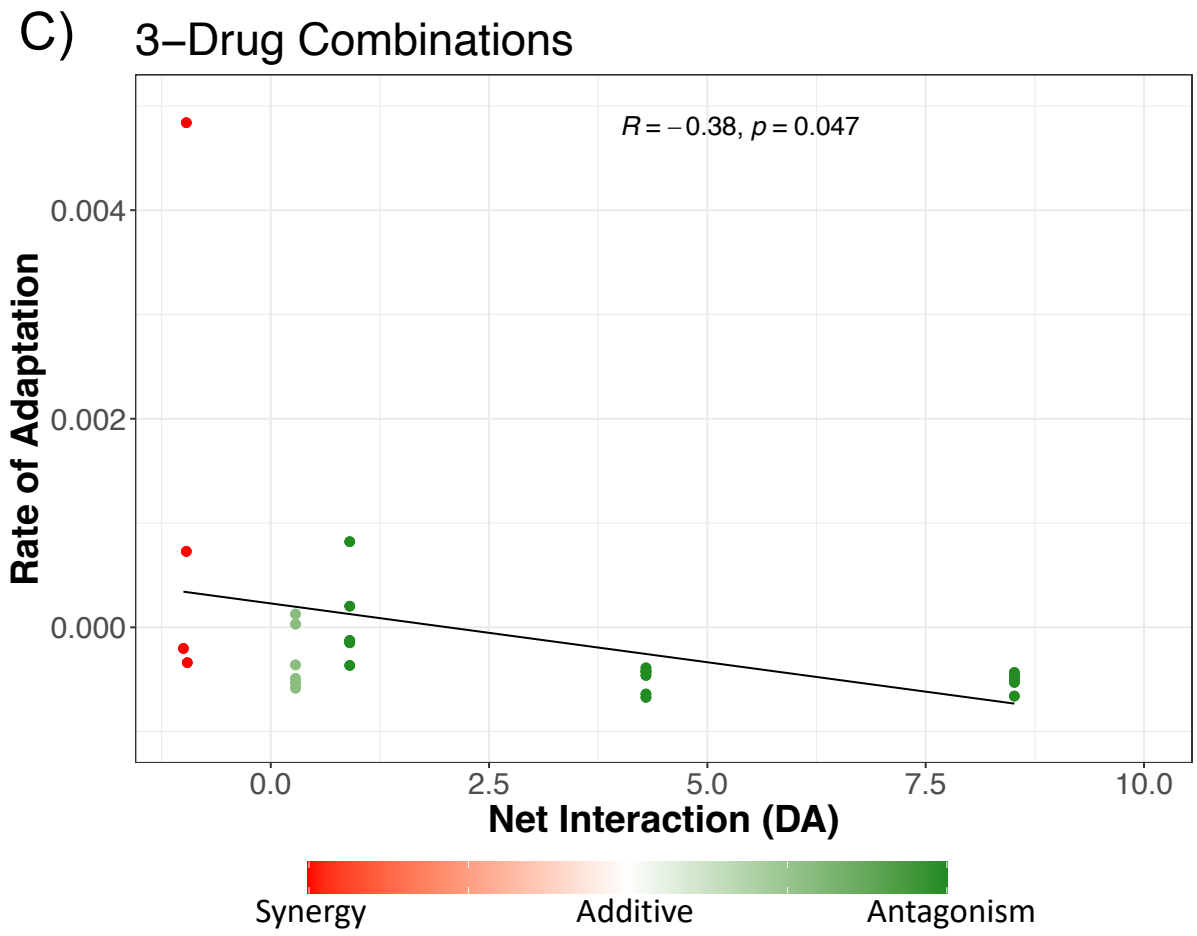


Figure 5-2. Synergistic combinations of two- and three- drugs correlate with faster rates of resistance adaptation. *A)* A Pearson correlation was performed on the corrected rates of adaptation and the net interaction of the pooled combination data for both two-drug and three-drug combinations. There was a significant negative correlation (R= -0.23, p = 0.002) which indicated that as net interactions become synergistic there are faster rates of adaptation. This trend is also observed when only examining *B),* two- drug combinations (R= -0.17, p = 0.037) or *C),* three-drug combinations separately (R= -0.38, p = 0.047).

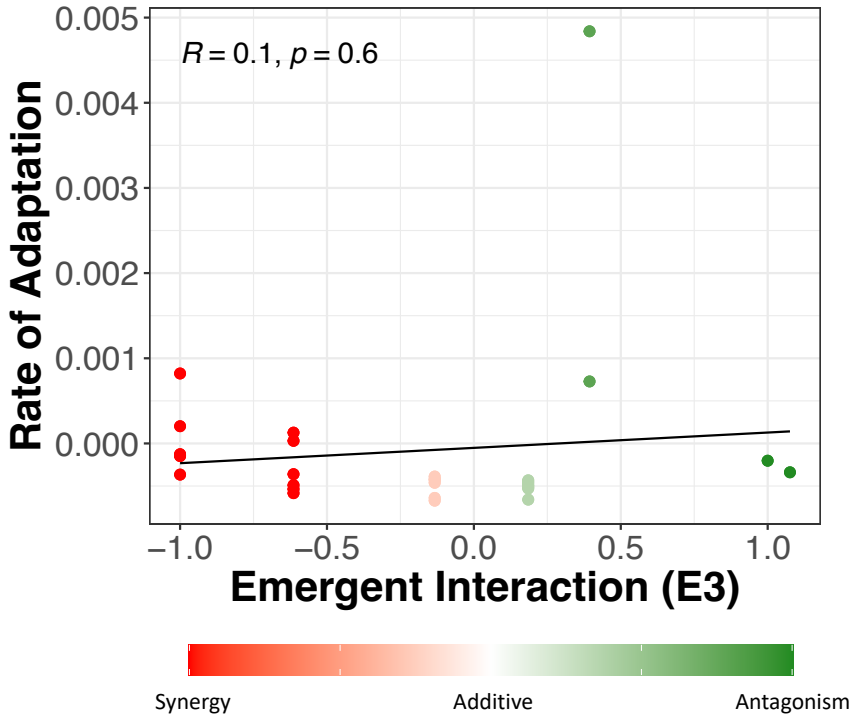


Figure 5-3. Emergent interactions do not correlate to rates of adaptation. A Pearson correlation was performed on the corrected rates of adaptation and the emergent interactions of the three-drug combinations. No significant correlation was found (R= 0.1, p= 0.6).

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