

Evolutionary dynamics of multidrug
antibiotic therapy in the model
organism *Pseudomonas aeruginosa*

Dissertation

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Declaration

I, **Ashley Gedon**, declare that:

Apart from my supervisor's guidance, the content and design of the paper is all of my own work;

The thesis has not already been submitted either partially or wholly as a part of a doctoral degree to another examining body and has not been published or submitted for publication;

The thesis has been prepared subject to the Rules of Good Scientific Practice of the German Research Foundation;

No academic degree has been withdrawn from me.

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Summary

The emergence and dissemination of antibiotic resistance among microorganisms poses a dramatic threat to modern medicine. With the increasing presence of multi-drug resistant bacterial infections, medical doctors are running out of effective drugs quicker than scientists are able to produce them. The lack of effective drugs threatens the functioning of modern medicine, as we are in danger of entering into a “post-antibiotic era” in which doctors are not able to treat even the simplest infections, and complex medicine including cancer treatment, surgery, and transplants is no longer a possibility. At the root of this problem is the enormous ability of bacteria to quickly and easily adapt to virtually any encountered toxin. Understanding the mechanisms behind bacterial evolution in response to various selective pressures is thus essential to combatting the antibiotic resistance crisis. Using evolutionary principles to optimize the deployment of currently effective antibiotics via rational treatment design has been used as a tool to select against antibiotic resistance. Administering antibiotics cyclically or in combination are two alternative regimens that have been suggested to have the potential to slow down or to decrease the selective advantage of resistance evolution in specific circumstances. However, these specific circumstances are not yet agreed upon, and the nature of the reduction in resistance seen with these therapies needs further experimentation. In this study, I aimed to optimize cycling antibiotic therapy with the Gram-negative human pathogen *Pseudomonas aeruginosa*. Using *in vitro* evolution experiments, the effects of cycling antibiotics, drug combinations, and a new regimen, termed cycling-in-combinations, on the evolution of drug resistance, were investigated. I found that cycling drugs in combination generally produced the highest extinction rates, the highest bacterial inhibition throughout evolution, and no significantly higher fitness costs when compared to cycling monotherapies and combination regimens. I additionally found that cycling-in-combination therapies showed minimal multidrug resistance in comparison to the respective cycling and combination therapies. Taken together, these results indicate that cycling drugs in combination could be a successful alternative antibiotic therapy that both eradicates large numbers of bacteria, but importantly, also limits bacterial resistance evolution.

Zusammenfassung

Das vermehrte Aufkommen und die stetige Verbreitung von Antibiotikaresistenzen stellt eine Bedrohung für die moderne Medizin dar. Die Zahl der Infektionen, die durch multiresistente Bakterien verursacht werden steigt rapide und Mediziner stehen inzwischen vor dem Problem, dass effektivere Medikamente und neue Wirkstoffklassen nicht schnell genug entwickelt werden. Die Gefahr besteht in eine post antibiotische Ära einzutreten, bei der selbst einfache Infektionen nicht mehr kausal behandelt und komplexe medizinische Behandlungen, wie die von Krebserkrankungen, aber auch Operationen oder Transplantationen, durchgeführt werden können. Die Ursache für das Problem ist dabei die einzigartige Fähigkeit von Bakterien, sich schnell an schädliche Umweltbedingungen anpassen zu können.

Ein Verständnis der Mechanismen, die hinter der bakteriellen Evolution als Antwort auf Selektionsdruck stehen, ist essentiell um den zunehmenden Antibiotikaresistenzen gegenüberzutreten. Von großer Bedeutung ist dabei die Optimierung des Einsatzes von zur Zeit wirksamen Antibiotika anhand evolutionärer Prinzipien und unter Zuhilfenahme rationaler Therapieschemata. Als alternative Behandlungsmöglichkeiten um die Selektionsvorteile von Antibiotikaresistenzen zu verringern oder zu verlangsamen, werden dabei entweder zyklische Wechsel verschiedener Wirkstoffe oder Kombinationstherapien vorgeschlagen. Die Art und Weise wie diese Therapien zu einer Verringerung der Antibiotikaresistenz führen, benötigt jedoch weitere wissenschaftliche Untersuchungen. Ziel dieser Arbeit war eine Optimierung der periodischen Antibiotikatherapie gegen das Gram-negative humanpathogene Bakterium *Pseudomonas aeruginosa*. Unter Zuhilfenahme von *in vitro* Evolutionsexperimenten wurde der Effekt der zyklischen und kombinierten Antibiotikabehandlung, sowie einer neuen Form, genannt zyklische Kombinationstherapie, auf die Evolution von Resistenzphänotypen getestet. Dabei habe ich festgestellt, dass die zyklische Kombinationstherapie zu der höchsten Bakterizidie und der höchsten bakteriellen Inhibierung durch Evolution, jedoch zu keinem signifikant höheren Fitnessaufwand, im Vergleich zu zyklischen Monotherapien und Kombinationstherapien, führt. Zudem kam es durch zyklische Kombinationstherapien nur zu einem geringen Aufkommen von multiresistenten Bakterien. Zusammenfassend zeigen diese Ergebnisse, dass die zyklische Kombinationstherapie eine erfolgreiche Alternativbehandlung darstellen kann, da dadurch zum einen die Anzahl an Bakterien sehr stark reduziert und zum anderen die bakterielle Resistenzentwicklung begrenzt werden kann.

Introduction

During this PhD project, I studied how multidrug therapies contribute to the evolution of resistance in the model organism *Pseudomonas aeruginosa*. I was specifically interested in evaluating the evolutionary consequences of antibiotics administered temporally differentially. I focused on comparing the treatment efficacy of antibiotics given as drug combinations, cycles of drugs, and a novel therapy that cycles drug combinations. Below, I will discuss the importance of antibiotics to our current lifestyle, how the urgent problem of multidrug resistance came to be, the unrivaled potential of bacterial adaptation, the phenomenon of bacterial intrinsic resistance, and finally, how incorporating evolutionary principles in treatment design could assist in our fight against antibiotic resistance.

Antibiotics as the bedrocks of modern society

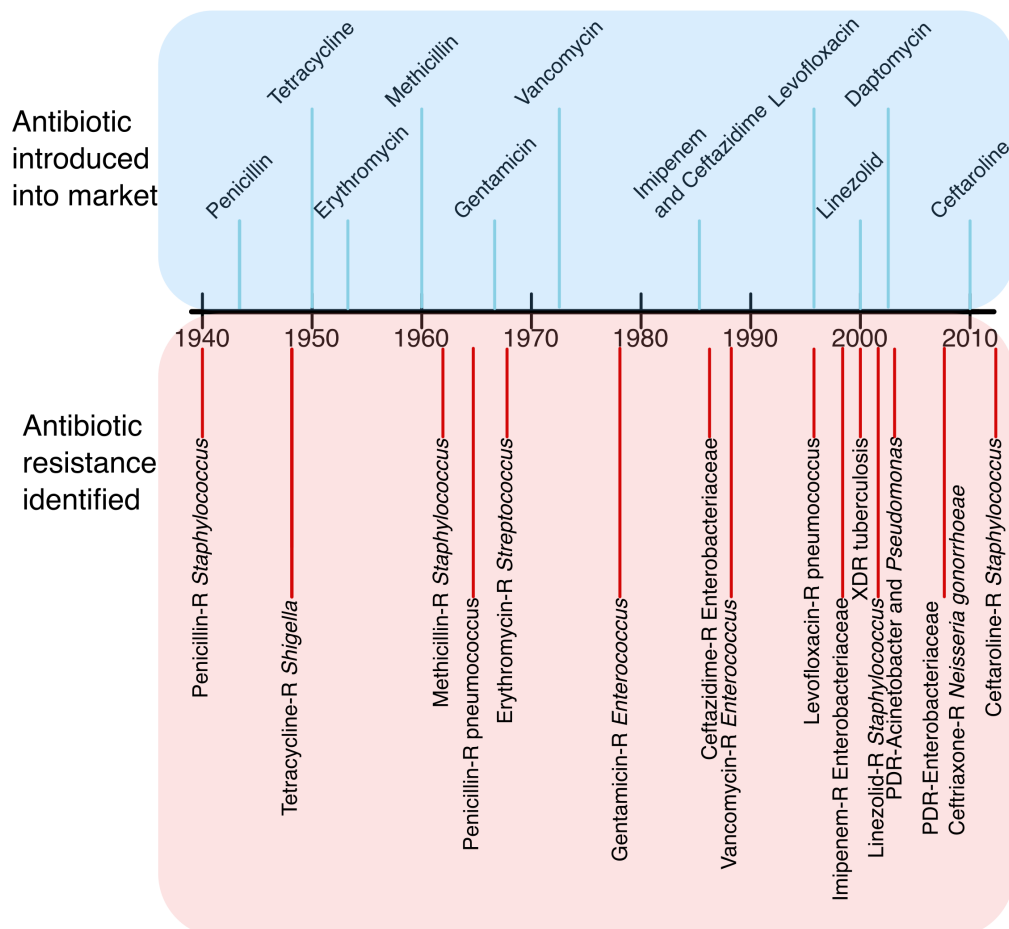
The importance of antibiotics in modern day life is difficult to fully comprehend, as they are so abundantly used in the clinical, agricultural, aquacultural, and horticultural industries. The commercial production of Prontosil in the 1930s, followed by penicillin in the 1940s, proved to be a critical turning point for modern medicine¹. Clinicians, previously having virtually no control over the outcome of an infection, were suddenly able to successfully treat most cases of formerly fatal diseases². Mortality due to bacterial infections decreased a staggering 60-75%³, allowing for a significant increase in human lifespan. The major cause of death subsequently shifted from infectious diseases to non-communicable diseases like cancer, cardiovascular disease, and stroke⁴. The introduction of these “wonder drugs” not only enabled the successful treatment of both mild and life-threatening bacterial infections, but additionally facilitated the development of complex medicine. Today, common and complex surgeries⁵, caring for preterm neonates⁶, cancer treatment⁷, and organ transplantation⁸ are all only possible with the prophylactic use of antibiotics protecting immunocompromised patients from developing life-threatening infections.

While these drugs were first identified and applied to treat serious bacterial infections, the therapeutic application of antibiotics currently accounts for less than half of all the total amount of antibiotics used commercially⁹. The agricultural industry is highly dependent on antibiotics, as these drugs are used both therapeutically as well as prophylactically as a means of growth promotion¹⁰. Over 10,000,000 kilograms of antibiotics were used for farm animals in the US alone in the year 2017¹¹, and it has been estimated that ~80% of the annual antibiotic usage in the US is consumed by farm food animals¹⁰. Aquaculture, a rapidly growing industry with relatively scant enforcement of drug usage, also relies on the use of prophylactic and therapeutic antibiotics to maximize profit by housing many fish in a small volume¹². Horticultural industries apply antibiotics to hectares of fields to prevent expensive crop yields from succumbing to bacterial diseases¹³. The presence of antibiotics in daily life is unavoidable, as their abundance in agricultural, aquacultural, and horticultural industries is accompanied by their domestic usage in cleaning products, food preservation, bee keeping, and preserving building materials from contamination¹⁴.

Our dependence on antibiotics unfortunately comes with a hefty price tag. Antibiotics act as a classical Darwinian selective pressure, in that the more they are used, the more selective pressure they exert, driving the evolution bacterial resistance. Although these drugs have only been in use for ~80 years, the millions of metric tons that have been and continue to be dumped into the environment fuel the selection and expansion of antibiotic resistance¹⁵⁻¹⁷. Today, antibiotic resistance is considered one of

the most pressing threats to global health¹⁸. The prevalence of bacteria resistant to single and multiple drugs is on the rise^{19,20}, and with this rise comes the concomitant decrease in available treatment options for patients suffering from these resistant infections^{21,22}. Antibiotic resistance currently causes 23,000 deaths annually in the US alone¹⁹. Bacteria resistant to multiple drugs are of particular concern, as physicians are left with either extremely limited or, in the worst cases, no possible treatment options^{19,23,24}. A total of 25,000 deaths are attributed to multidrug resistance (MDR) in the EU annually²⁵. While these numbers may seem comparably small, if current resistance trends continue, antibiotic resistance will be the leading cause of death by the year 2050, claiming an annual 10 million lives, and outnumbering deaths attributed to cancer and road accidents combined²⁶. Without functioning antibiotics, the rate of postoperative infection is 40-50%, with 30% of those infections proving to be fatal, making even the simplest of surgeries likely to fail²⁷. We are currently in danger of heading back into a pre-antibiotic era, where clinicians lack the ability to treat even the simplest infections, and the ability of hospitals to perform complex surgeries, care for prenatal infants, and perform chemotherapy will be lost²⁸.

Many factors contribute to how and why we have arrived at the cusp of a pre-antibiotic era²⁹. The period following the distribution of penicillin in 1940s was termed the 'golden age of antibiotic discovery', in which new antibiotic classes were being continuously introduced into the pharmaceutical market. During this time, most of the currently-effective and medically relevant antibiotic classes were characterized¹⁹, and experts hypothesized that they had effectively eradicated infectious diseases³⁰. Although resistant bacterial strains were, in fact, present during this time (Fig. 1), the constant introduction of novel drug classes and drug modifications kept the bacteria temporarily at bay, and experts hopeful that infectious diseases were a problem of the past^{9,31}. Today, however, the antibiotic pipeline has run dry and there has been a 90% decrease in antibiotic approvals in the last 30 years². It has been hypothesized that the lack of novel antibiotics in combination with the extreme overuse of these drugs, specifically in the medical and agricultural industries, has contributed significantly to the current antibiotic crisis^{23,25,26}.



PDR = pan-drug resistant, R = resistant; XDR = extensively drug resistant

Dates are based upon early reports of resistance in the literature. In the case of pan-drug resistant *Acinetobacter* and *Pseudomonas*, the date is based upon reports of health care transmission or outbreaks. Note: penicillin was in limited use prior to widespread population usage in 1943.

Figure 1. Timeline of antibiotic discovery and subsequently observed resistance. The top axis shows the introduction of select antibiotics into the market, and the bottom shows first reported clinical resistances and the organisms boasting said resistance. The scheme was adapted from a report released by the Center for Disease Control in 2013¹⁹.

The unrivalled mastery of bacterial evolvability

When summarizing Darwin's work, Megginson wrote, "According to Darwin's *Origin of Species*, it is not the most intellectual of the species that survives; it is not the strongest that survives; but the species that survives is the one that is able best to adapt and adjust to the changing environment in which it finds itself."³² Bacteria's capacity to adapt to diverse environments is unparalleled, as various bacterial species have been isolated in virtually every known environment^{33,34}. Bacteria not only survive, but have been found to thrive in extremely harsh environments where few other organisms are able to persist, including environments with high pressures, pH stress, osmotic stress, and temperature stress^{33,35}. Not only are bacteria able to endure a wide variety of environments, but they are also able to endure extreme fluctuations within their environment³⁶⁻³⁸, further highlighting their evolvability. It is therefore not surprising that this evolvability translates to their ability to develop antibiotic resistance to a broad range of toxic drug environments. The Antibiotic Resistance Genes Database was recently created by scanning the literature for publicly available bacterial antibiotic

resistance genes, and identified over 20,000 diverse resistance genes, corresponding to abundant evolutionary paths to resistance³⁹. With so many alternative paths leading to resistance, it is unastonishing that resistance has been reported for each of the 15 classes of antibiotics currently on the market²⁴.

In order to understand how and why bacteria evolve resistance to antibiotics, it is first necessary to understand the nature and origin of antibiotics. The term ‘antibiotic’ currently refers to a broad spectrum of structurally and functionally diverse compounds, but can be generally defined as a natural or synthetic molecule that inhibits or kills bacteria⁴⁰. First coined by Waksman, the word ‘antibiotic’ means ‘against life’, and was originally suggested as there was some discussion on how to refer to these sometimes strikingly different chemical compounds that had one thing in common—their ‘injurious effect’ on microbial growth⁴⁰. Although chemically diverse, these compounds can be roughly classified based on pharmacodynamics. Bactericidal antibiotics directly kill bacterial cells, while bacteriostatic drugs prevent further bacterial replication, relying on the host’s immune system to clear the remaining, non-dividing cells⁴¹. A drug is classified as bacteriostatic if after treatment with a high dose of antibiotic (4x the minimum inhibitory concentration (MIC)) for 18-24 hours results in 0.1% of the treated cells being able to subsequently resume growth. Consequently, antibiotics are classified as bactericidal if more than 99.9% of treated cells subsequently cease to divide⁴². Although it has been shown that classification as bactericidal or bacteriostatic can depend on the bacterial density, growth conditions, antibiotic concentration, and various other host responses⁴³, other studies have concluded there is indeed a difference in the “cidality” of the drugs, arising from differing binding capacities in the ribosome⁴⁴. Antibiotics can be further classified based on the number of organisms they affect (broad or narrow spectrum antibiotics), their mechanism of action, and their chemical structure. Antibiotics target specific bacterial metabolic pathways, including cell wall synthesis, protein synthesis, DNA or RNA synthesis, and folate synthesis (Fig. 2A)⁴¹. Antibiotic classes further sort drugs based on their chemical structure. For example, beta-lactam antibiotics all contain a beta-lactam ring, and target cell wall synthesis by binding to penicillin binding proteins, rendering the bacterium unable to form the cell wall. However, there are numerous drugs inside the class of beta-lactams, all of which have slightly different structures and corresponding molecular targets.

Although anthropogenic activities have quickened the spread of antibiotic resistance, it is important to note that antibiotic resistance is not a novel phenomenon, but is instead a natural response for survival, and a classic example of “survival of the fittest”⁴⁵. Antibiotic resistance has been present long before the commercial production and dissemination of antibiotics. Resistance genes have been found in permafrost soil samples dating from over 30,000 years ago⁴⁶, in cave microbiomes over 4 million years old⁴⁷, and in the gut microbiome of mummies carbon dated from 980-1170 A.D.⁴⁸. This is because most medically relevant antibiotics are or have been derived from metabolites produced by bacteria and fungi, the majority coming from the soil-dwelling bacteria Actinomycetes⁴⁹. Bacteria and fungi produce these compounds as a sort of chemical warfare, which inhibits growth of neighboring competitor species, thus securing more of the surrounding environmental resources⁵⁰. Antibiotics can additionally act as signaling molecules between both similar and diverse bacterial species, allowing the bacteria to alter their metabolism in response to environmental signals^{50,51}. Organisms producing and releasing antibiotics into the environment must have some sort of protection from the toxic chemical being released, or the release of the drug would result in a suicide mission. This is, again, done with ease, as a recent

study looking at the resistomes of soil-dwelling bacteria found that every strain tested was resistant to an average of 7-8 antibiotics, with some strains resistant to up to 15 of the 21 tested drugs⁵².

Bacteria have been evolving antibiotic resistance for billions of years, and, as outlined above, are proficient in adapting to a myriad of toxic environments. The fluidity of the microbial genome is key to its accomplished evolvability, with resistance evolving by either spontaneous mutations or horizontal gene transfer (HGT). Spontaneous mutations are an essential part of any organism's ability to evolve, and mutation rate has been shown to increase when bacteria are exposed to environmental stressors^{53,54}. This increased rate of spontaneous mutations can and does lead to detrimental, deleterious effects for the bacteria^{55,56}, but can also importantly lead to beneficial mutations which help the cells cope with the surrounding drug toxicity⁹. Spontaneous mutations occur on the bacterial chromosome, and confer resistance in one of three ways: (1) minimizing the amount of drug in the bacterial cell either by decreasing penetration, by downregulating or removing porins, or by active efflux of the drug; (2) modifying the antibiotic target so that the drug will no longer be able to recognize its intended target, or by modifying the expression level of said target; (3) modification of the antibiotic itself by an actively produced enzyme (Fig. 2B)⁵⁷⁻⁵⁹.

HGT is another mechanism that can quickly confer resistance to a multitude of drugs, and has been shown to play a large role in the spread of antibiotic resistance⁶⁰. Bacteria are capable of taking up foreign DNA from the surrounding environment, which allows the rapid diversification of their otherwise clonal gene pool. This process of DNA trading enables bacteria to rapidly gain MDR from both phylogenetically similar and diverse species⁶¹. HGT classically takes place in one of three ways: (1) transformation occurs when a bacterial cell is able to incorporate foreign DNA into the chromosome and express the novel gene; (2) transduction requires the help from a phage, which mobilizes genetic elements, then preys on bacteria, thus transferring said genetic elements from cell to cell; (3) conjugation requires the direct contact between two bacterial cells, and transfers genetic packages via a pilus^{59,60,62}.

A plethora of possibilities exist for bacteria to gain resistance to one or multiple drugs. Once resistance is gained, cells are able to grow at high drug concentration, which is otherwise toxic for sensitive cells. The application of an antibiotic to a bacterial population containing resistant cells thus selects for the resistant clones, propagating resistance genes and thus directly selecting against the sensitive clones⁵⁸.

Since the mutations associated with antibiotic resistance all involve important cellular machinery, these mutations may also confer a cost for the harboring bacteria. Indeed it has been shown that most resistance mutations show an associated fitness cost in the absence of antibiotic pressure for a variety of drugs and in a variety of bacterial species for resistance acquired both by spontaneous mutation⁶³⁻⁶⁸ and horizontal gene transfer^{69,70}. While the extent of the cost varies substantially based on species and associated drug to which resistance was evolved, common costs include decrease in growth rate, decrease in competitive ability, reduced transmission rate, and decrease in virulence^{63,71-73}. These associated fitness costs are pivotal in understanding and combatting antibiotic resistance, as they impact the rate and extent of resistance transmission⁷⁴⁻⁷⁶. Importantly, additional studies have failed to find associated fitness costs from antibiotic resistance^{77,78}, and even, in some cases, result in increased fitness compared to the sensitive strain^{79,80}. The presence and rate of compensatory evolution seems, in large part, to depend on the severity of the fitness cost⁸¹. However, presence of compensatory mutations have been identified in multiple bacterial species^{79,82,83}.

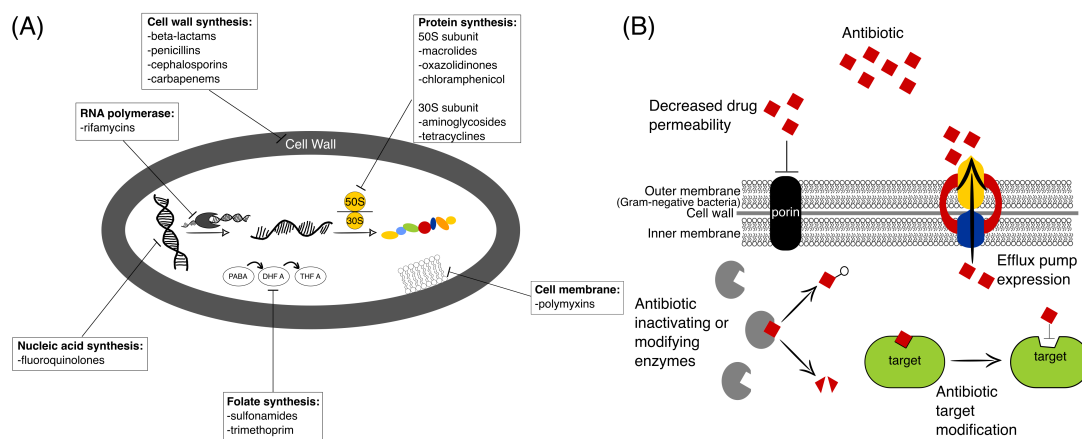


Figure 2 Targets and mechanisms of antibiotic resistance. (A) Common targets of currently effective antimicrobial agents and respective the antibiotics classes that target the generalized bacterial metabolic process. The figure was adapted from Brown, 2015⁸⁴. (B) Common mechanisms of antibiotic resistance including decreased drug penetration, efflux pump expression, antibiotic target modification, and antibiotic inactivating or modifying enzymes. The figure was adapted from Andersson *et al*, 2016⁸⁵.

Additional paths to antibiotic resistance

The ability to evolve drug resistance is coupled in some bacterial species with an intrinsic resistance to particular antibiotics. Intrinsic resistance is universally exhibited by a species, present not because of antibiotic selective pressure or horizontal gene transfer, but because it is the ancestral phenotype⁸⁶. An intrinsic resistome functions by increasing the MIC necessary to inhibit bacterial growth, and can be defined for all bacterial species, although not all species are defined as ‘intrinsically resistant’ in respect to clinical breakpoints^{86,87}. Isolates are classified as ‘intrinsically resistant’ to a drug if they present an increased MIC relative to the breakpoint concentration shown to inhibit bacterial growth during an infection⁸⁸.

Lack of antibiotic target, active expression of chromosomally encoded antibiotic-inactivating enzymes, outer membrane impermeability, and efflux pump expression are common phenotypes stemming from the intrinsic resistome^{89–91}. Gram-negative bacteria are notorious for their multidrug intrinsic resistance. The structure of their impermeable outer membrane restricts many drug classes that are otherwise effective on Gram-positive bacteria from entering the Gram-negative cell⁸⁹. The presence of multiple actively-expressed, chromosomally-encoded efflux pumps expels drugs that are able to cross the outer membrane, which further reduces intracellular drug concentration⁹⁰. The intrinsic resistance of Gram-negative bacteria was originally attributed to the inability of the drug to efficiently permeate and remain in the cell. However, recent studies on bacterial intrinsic resistomes have highlighted that intrinsic resistance involves a variety of genes not associated with membrane permeability and efflux^{87,92,93}, suggesting that the intrinsic resistance is more complexly ingrained in bacterial DNA than previously anticipated. This intrinsic resistance not only significantly reduces treatment options, specifically for Gram-negative infections, but also has been associated with an increased ability to gain high levels of genetically-encoded resistance⁹³, making these pathogens even more difficult to eradicate.

Phenotypic resistance is similar to intrinsic resistance, in that it does not require additional genetic modifications, but also requires bacterial cells to take environmental cues and to respond accordingly. Phenotypic resistance is a non-heritable, transient

form of resistance, and includes bacterial biofilm formation, swarming motility, and cell growth retardation or cessation⁹⁴. Biofilm formation is characterized by a bacterial switch from planktonic growth to forming aggregates that are surrounded by a matrix of extracellular polymeric substance⁹⁵. Biofilm formation poses a particular threat for patients, as once established, biofilms are notoriously difficult to eradicate and often result in persistent infections^{96,97}. Living in these cooperative formations poses two great advantages. Firstly, biofilm growth increases tolerance to antibiotics, allowing bacterial cells to withstand up to 100-1,000 times higher drug concentrations than planktonic cells^{96,98,99}. Secondly, biofilm growth impairs the ability of host innate immune defenses, making the successful identification and eradication of the foreign pathogens in the body more difficult¹⁰⁰. In contrast to biofilm formation, swarming motility involves the formation of hyper-flagellated cells, which can rapidly move to nutrient-dense environments, and tolerate high doses of antibiotics¹⁰¹.

Bacteria can also evade antibiotic killing by transitioning to a dormant phenotype, which either slows or completely arrests bacterial division. As bactericidal antibiotics require active growth to effectively kill targeted bacteria, slowing cell division allows these bacteria to transiently survive exposure to high concentrations of antibiotics without acquiring the associated genetic resistance^{102,103}. This can be achieved by slowing replication, referred to as tolerant cells, or by completely stopping growth, referred to as persistent cells¹⁰⁴. The deceleration of replication exhibited by tolerant cells correspondingly increases the length of time a drug needs to be applied to achieve killing, regardless of the applied drug concentration¹⁰⁵. Bacterial persistence refers to a subpopulation of bacteria in a clonal population that are not eradicated by high concentrations of an antibiotic, but persist in this dormant state as long as the drug is present^{106,107}. Bacterial persistence and tolerance are problematic as they enable bacteria to transiently survive in toxic environments without the associated resistance mechanisms and thus without fitness costs. Additionally, both phenotypic resistance mechanisms have been shown to be correlated with an increased likelihood of acquiring genetic resistance^{103,108}.

As outlined above, resistance to antibiotics is a large problem, with numerous paths to a similar outcome—the ability to grow and thrive in previously toxic environments. However, not all bacterial species are equally equipped to survive antibiotic treatments. Select species are particularly distinguished in surviving antibiotic treatment by one or more of the aforementioned pathways, and impose a particular burden on healthcare systems. *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter*, also known as the ESKAPE pathogens, have been identified as the leading cause of nosocomial infections¹⁰⁹. Finding effective treatment options is difficult for clinicians, as these species are often intrinsically resistant as well as able to acquire genetic resistance to multiple if not all possible antibiotics^{109–111}. This multidrug resistance leaves clinicians with extremely limited treatment options, forcing doctors to prescribe older, toxic, “last resort” drugs including carbapenems and polymyxins, which can be harmful to patient health¹¹². Even with the use of last resort drugs, the treatment options are dismal, as the sheer presence of an infection caused by an ESKAPE pathogen is associated with increased patient mortality¹¹¹. The World Health Organization (WHO) released a similar list of pathogens which are in critical, high, and medium need for research and development¹¹³. Present in the critical priority list are carbapenem-resistant *Acinetobacter baumannii*, carbapenem-resistant *Pseudomonas aeruginosa*, and carbapenem and 3rd generation cephalosporin-resistant Enterobacteriaceae. Gram-negative pathogens dominate these lists, both because of

their higher rates of intrinsic and genetically acquired resistance and because the last developed and introduced novel antibiotic effective against Gram-negative infections were the quinolones, discovered in 1962¹¹⁴. *Pseudomonas aeruginosa*, a Gram-negative pathogen that has earned a place on both aforementioned lists has been described as one of the most successful nosocomial pathogens¹¹⁵, and is the main subject of this dissertation.

***Pseudomonas aeruginosa* as a model organism for studying antibiotic resistance**

Pseudomonas aeruginosa (PA) can be isolated from a wide range of environments ranging from soil and water, but can also act as an opportunistic pathogen in plants, animals, and humans³⁸. First described in the 1850s, PA was particularly problematic as a wound infection, where it produced a distinctive colored blue-green puss in patient's bandages¹¹⁵. Freeman later described the infections caused by PA as, "a serious disease, often resulting in fatality, although recovery occasionally takes place"¹¹⁶. Intrinsically resistant to many antibiotic classes, PA has low outer membrane permeability¹¹⁷, constitutive expression of multiple efflux pumps¹¹⁸, and chromosomally-encoded antibiotic modifying enzyme beta-lactamase^{119,120} that contribute, but are not solely responsible for, its intrinsic resistance^{87,121,122}. In addition to the impressive array of intrinsic resistance, PA boasts one of the largest bacterial genomes (strains vary from 5.5-7 million base pairs, Mbp)^{123,124}, nearing the genomic complexity of the eukaryotic model organism *Saccharomyces cerevisiae*¹²³, and is considerably larger than the genomes of other ESKAPE pathogens (genome sizes range from ~2.8-5.3 Mbp with some strain variability¹²⁵⁻¹²⁹). The largest proportion of regulatory genes and networks found in a bacterial species accompanies the large genome of PA¹²³, providing multifold ways to intrinsically and adaptively respond to environmental stressors.

The natural versatility PA possesses is conveyed in the diversity of diseases the organism causes, infecting virtually every tissue, and causing infections ranging from urinary tract infections, surgery/transplantation tissue infections, and respiratory infections, to sepsis¹³⁰⁻¹³². Primarily infecting immunocompromised individuals, PA is a major cause of nosocomial infections worldwide^{133,134}, and is particularly problematic in respiratory infections, being the leading cause of ventilator associated pneumonia in the US and Europe¹³⁵⁻¹³⁷. PA also causes chronic lung infections in patients suffering from cystic fibrosis (CF), and is the main cause of morbidity and mortality for this disease^{138,139}. CF is an autosomal recessive disease that emanates in a thick mucus accumulating in many areas of the body, resulting in an optimal environment in which PA can proliferate and hide from immune responses and antibiotic therapies alike¹⁴⁰⁻¹⁴³. Although the microbial ecology of the CF lung hosts a wide variety of bacterial species¹⁴⁴, PA's presence is associated with deteriorating patient health^{145,146}, and is very unlikely to be eradicated once detected^{139,147}. A positive PA swab routinely results in an intermittent infection, a temporary "clearance" period, and ultimately results in chronic infection^{97,148}. A chronic PA infection will most likely lead to respiratory failure, lung transplant, or mortality for CF patients¹⁴⁷. The difficulty in eradicating an established PA infection has been connected to a high number of bacterial PA persister cells¹⁴⁹, extensive biofilm formation^{150,151}, a high degree of phenotypic variability¹⁵², and a high incidence of MDR¹⁵³. CF is an unfortunate, but extraordinary example of PA's evolutionary capabilities, as these organisms are not only able to grow in the harsh lung environment, but are also able to withstand chronic exposure to antibiotics while continuing to evolve and proliferate.

PA's ability to intrinsically resist many antibiotics and to acquire additional genetic resistances results in very few, if any, treatment options for infections caused by this organism²⁵. PA's intrinsic resistome includes many beta-lactam antibiotics, older quinolones, chloramphenicol, tetracycline, macrolides, trimethoprim-sulfamethoxazole, and rifampicin¹⁵⁴. Currently effective anti-pseudomonal drugs are shown in Table 1^{130,155,156}. However, widespread resistances to many of these drugs are rising, as a recent report in the EU identified over 30% of all tested PA as being resistant to at least one of the important anti-pseudomonal drugs¹⁵⁷. Multidrug resistance further reduces remaining treatment options, and the presence of MDR PA is significantly correlated with increased mortality rate¹⁵⁸. Rates of MDR are similarly escalating as ~15% of tested PA isolates displayed resistance to at least 3 tested drugs in Europe¹⁵⁶, 37% of PA infections showed MDR in Brazil¹⁵⁹, and an astounding 54% of isolates were MDR in China¹⁶⁰. Although HGT has been observed to confer resistance to aminoglycosides and additional beta-lactam antibiotics in PA^{161,162}, chromosomal mutations seem to be the major driver of resistance evolution in PA^{115,163}, suggesting that the evolutionary dynamics of this organism are key to understand its extraordinary capacity to resist antibiotic killing.

Table 1 Currently active anti-pseudomonal drugs and corresponding bacterial targets¹⁵⁶.

Currently-active anti-pseudomonal drugs	Class	Functional Target
Ciprofloxacin Levofloxacin	Fluoroquinolones	DNA synthesis
----- Gentamicin Tobramycin Amikacin	Aminoglycosides	Protein synthesis
----- Piperacillin+tazobactam Ticarcillin+tazobactam Ceftazidime Cefepime Ceftolozane+tazobactam Imipenem Doripenem Meropenem	Beta-lactams Cephalosporins Carbapenems	Cell wall synthesis
----- Polymyxin B Colistin	Polymyxins	Outer membrane disruption

Using evolutionary principles to design antibiotic treatments that select against multidrug resistance

It is clear that antibiotic resistance is of great concern and that actions must be taken to control this threat. As mentioned above, resistant strains have long complicated the success of antibiotic therapy^{164,165}. However, several strategies have kept the medical community afloat, allowing the majority of bacterial infections to continue to be successfully eradicated. The introduction of new drugs into the market is a major contributor to the ongoing functioning of modern medicine¹⁶⁶⁻¹⁶⁸. The search for novel antibiotics continues, with scientists now desperate to isolate or synthesize

antimicrobial compounds¹⁶⁹. The modification of currently effective antibiotics has also had substantial success in extending the shelf life of older antibiotics. For example, the mutation that allows bacteria to resist aminoglycosides is primarily an enzymatic region-specific modification¹⁷⁰. The newer generations of aminoglycosides, including tobramycin, lack the site to which the enzyme previously bound, removing one of the primary paths bacteria possess to acquire resistance¹⁷¹. The addition of an adjuvant is another widely used mechanism to prolong the use of our current antibiotic arsenal. For instance, beta-lactam antibiotics are susceptible to degradation by one of over 850 currently identified beta-lactamase enzymes¹⁷². Distributing a beta-lactam antibiotic in combination with a beta-lactamase inhibitor, an enzyme that does not allow the beta-lactamase to cleave the antibiotic beta-lactam ring, renders the previously ineffective antibiotic, effective once more¹⁷³.

As the condition of our functioning antibiotic armory continues to worsen, unconventional suggestions to contain this epidemic have been proposed. Anti-virulence therapy suggests the removal of bacterial virulence will theoretically allow the host immune system to independently combat the bacterial infection^{174,175}. Efflux inhibition in combination with antibiotics provides restored selective antibiotic function as well as increases antibiotic penetration, consequently decreasing bacterial intrinsic resistance of some problematic species^{176–178}. Phage therapy has also seen some preliminary success, and involves fighting the bacterial infection with a virus that targets the infecting bacteria, but also comes with the associated fear of introducing an additional live pathogen into the human body^{179,180}. Antimicrobial peptides, an important component of the innate immune system, also show antimicrobial activity, and efficiently fight otherwise difficult-to-eradicate infections^{181–183}. Although each of these potential therapies presents many advantages, they are all susceptible to one very important Achilles heel—bacterial evolution. Regardless of the therapy, bacteria are still capable of evolving, and until bacterial evolution is better understood, creating new therapies will just be a temporary bandage on a bleeding wound.

Understanding bacterial evolution is crucial to being able to get to the root of the antibiotic resistance crisis. Experimental evolution has been used as a valuable tool for understanding evolutionary dynamics in many fields including cancer^{184–186}, pesticide resistance^{187,188}, bacterial,^{189–192} viral^{193–195}, and parasitic^{196,197} infectious diseases^{198–201}. As bacteria are easy to house, have a rapid doubling time, high mutation rate, and clonal populations, the evolution of these organisms can be seen in real time, which can aid in our understanding of how specific phenotypes arise by evolution^{202,203}. Evolution in terms of antibiotic resistance is a double-edged sword, as the selective pressure of drug treating the infection will undoubtedly eventually result in drug resistance^{29,31,204}. Several studies have demonstrated some level of predictability in terms of evolution of antibiotic resistance mutations, and their associated phenotypes^{205,206}. Others have contrastingly identified various and divergent paths toward resistance^{57,199,207}, and that the assembly of mutations towards resistance has been shown to be dependent on a myriad of lifestyles and environmental factors²⁰⁸. Understanding antibiotic resistance dynamics will help us further dissect how these organisms respond to changes in their environment, and ultimately allow the exploitation of this information to design more effective antibiotic treatments²⁰⁹.

With the inevitability of resistance, it is reasonable to wonder ‘what is the ultimate goal of an antibiotic treatment?’. While the previous goal of antibiotic treatment was to kill as many bacteria as quickly as possible, I question this widespread thinking and redefine treatment goal as a combination of killing capacity in addition to selecting against, or minimally slowing down of antibiotic resistance evolution.

However, until now, evolution has rarely been taken into account during antibiotic testing or clinical practice^{200,204}. A single drug, equating to a static environment, given over a standard duration of time (5-10 days) is still commonplace in antibiotic therapy^{210,211}. In some cases, antibiotic combinations are given as an exception for select, particularly difficult-to-treat diseases²¹². Deploying multiple drugs during a single therapeutic period has been suggested to increase environmental complexity and thus to decelerate bacterial evolution⁵⁸. The addition of multiple drugs into one environment results in the interaction of drugs either physiologically or evolutionarily (Fig. 3B,C)⁵⁸. When drugs are simultaneously combined during therapy, they can interact additively, synergistically, antagonistically, or suppressively (Fig. 3B)^{58,213–216}. Similarly, when a population displays an already-acquired resistance to one drug, and is subsequently challenged with an additional drug, hypersensitivity to (collateral sensitivity) or resistance against (collateral resistance) the second drug can be shown (Fig. 3C)^{215,217,218}. Each of these interactions can have beneficial as well as detrimental effects on the evolution of antibiotic resistance, but understanding these phenotypes can help us better understand the dynamics of bacterial resistance evolution in response to multidrug environments.

Combination therapy, or the direct addition of two or more drugs spatially has been in discussion for more than 80 years^{213,216,219}. The theory behind combination therapy lies in the simple assumption that the more drugs used to treat a single infection, the higher the selective pressure exerted on the bacteria. A higher selective pressure can eradicate the bacterial population quicker, and is more likely to treat an infection resulting from multiple bacterial species. Additionally, drug combinations decrease the likelihood of resistance evolution, as the drugs target multiple independent metabolic pathways²¹⁹. Clinicians have long favored synergistic drug combinations, as they achieve accelerated clearance by using a lower drug dose of potentially toxic antibiotics^{220,221}. However, until recently, the interaction of antibiotics was only measured over 24 hour periods of time, ignoring how these interactions were affected by evolution²²². Experimental *in vitro* evolution experiments have found that using synergistic combinations actually increases the rate of adaptation in *E. coli*^{222,223}, speeding up the evolution of drug resistance. The reasoning behind this faster rate of adaptation is that when a bacterium acquires drug resistance, the cell acts as though there is less of the associated drug present^{58,214,224}. In the synergistic drug environment, the drugs amplify each other's effects. Therefore, the aforementioned resistant cell now has the ability to resist one of the two antibiotics in the previously synergistic pair, and now behaves as if there is a lower concentration of that antibiotic in the environment. This consequently relieves the synergistic effect that was previously achieved by the high concentration of both drugs, and renders the second drug ineffective^{58,214,222,223}. In contrast, combinations displaying antagonistic or even suppressive interactions show delayed antibiotic resistance evolution^{214,225,226}. This delay in adaptation is thought to result from the fact that there is a sort of protection from the full potential of both of the drugs during antagonistic or suppressive drug combination treatment. When a bacterial cell acquires resistance to one drug, this protective effect is lost, resulting in the additional drug being able to exert its full effect on the remaining population^{58,214,226}. Evolutionary interactions also appear to play a role in rate of resistance evolution during combination therapy, as collateral sensitivity has been shown to decrease the rate of adaption during combination therapy in *P. aeruginosa*¹⁹⁸, *E.coli*²²⁷, and *S. aureus*²²⁸. Thus, physiological and evolutionary interactions have both been proven to be important in terms of resistance evolution in response to combination therapies.

However, a more complete understanding of how these interactions influence resistance evolution could help uncover how to more efficiently use drug combinations.

Cycling between drugs involves adding a temporal distance between the addition of the first and the second drug in a desired combination. This heterogeneous environment complicates resistance evolution, as the bacteria need to adjust to multiple, changing environments, and selective pressure differs for each drug. However, the terminology ‘antibiotic cycling’ is not always clear, and has been used to refer to different ways to cycle drugs. For example, hospitals refer to drug cycling as the scheduled rotation of antibiotics in a hospital ward used to house patients with a particular disease, in an attempt to limit the spread of drug resistance between patients (Fig. 3A). Switching between deployed drugs in a hospital, where immunocompromised patients are housed in close proximity, and guidelines dictate the antibiotic to use for a specified infection, would theoretically limit the spread of multidrug resistant pathogens between patients²²⁹. For example, all patients suffering from general burn wounds would be put on antibiotic A in May, and the drug of choice would switch to antibiotic B in June, thus removing the selective pressure applied by drug A and the associated resistance to said drug. Antibiotic mixing is an additional hospital-wide attempt to limit the transmission of drug resistance that gives patients suffering from similar diseases on differing drugs (Fig. 3A)²³⁰. The effectiveness of implementing these cycling and mixing drug strategies throughout an entire hospital is unclear, with some studies showing some success in limiting antibiotic resistance^{231–233} whilst others finding no noticeable differences^{234–236}.

While these studies focus on limiting the spread of resistance, the remainder of this thesis will focus on cycling drugs within one patient, which attempts to prevent the evolution of resistance in the first place (Fig. 3A). In contrast to cycling or mixing drugs within a hospital, cycling between multiple drugs during a single antibiotic therapy within a single patient has been suggested as a means to delay resistance evolution⁵⁸. This type of cycling therapy involves short exposures of the administered drugs, with drug switches taking place every 12-24 hours. Cycling drugs in this way has limited the evolution of resistance in *P. aeruginosa*^{201,237} and in *S. aureus*²³⁸, and was shown to outperform combination therapy in terms of bacterial inhibition in *E. coli* even at sublethal doses²³⁹. Evolutionary interactions also contribute to this alternative treatment’s efficacy, as the deceleration of resistance evolution depends on the presence of reciprocal collateral sensitivity in the component drugs in *E. coli*^{239,240} and in *S. aureus*²³⁸. These evolutionary interactions have been suggested as a means of designing rational antibiotic therapy that takes into account the probability of evolutionary outcomes when select drugs are applied and thus selects against multidrug resistance²⁴⁰. Importantly, recent studies have highlighted that collateral profiles of organisms belong to the same strain are not always identical, and that clones can evolve resistance to the same antibiotic in different ways, leading to differing collateral profiles^{199,207,241}.

Recently, there has also been renewed interest in a phenomenon called hysteresis that has been shown to have an effect on resistance evolution under antibiotic cycling²⁰¹. Cellular hysteresis describes a change in bacterial physiology in response to a very short period of antibiotic exposure, which would not normally not elicit killing. Aforementioned changes in bacterial physiology in turn cause increased or decreased susceptibility to the second drug in the cycling regimen. The presence of negative hysteresis, in which the short application of the first drug in the cycle increases susceptibility to the second drug, was shown to increase extinction and reduce resistance rates in *P. aeruginosa*²⁰¹.

Throughout this introduction, I have discussed how antibiotic resistance is a dire situation, urgently in need of further research and understanding. I have additionally outlined how and why bacteria are so highly adaptable to antibiotic environments. Although there are many novel and exciting ideas in terms of combatting antibiotic resistance, I have argued that evolution is at the core of every possible treatment alternative, and no matter the agent, bacteria will eventually evolve resistance. Therefore, understanding bacterial evolutionary dynamics will give us the tools to define areas of bacterial weakness and to combat antibiotic resistance evolution. Combining drugs and cycling between drugs have both been shown to decelerate the rate of resistance evolution, however the manner in which they achieve this reduced rate is dependent on both physiological and evolutionary interactions. Knowing this information, I aimed to test a novel alternative antibiotic treatment, which cycles drug combinations. Cycling-in-combinations adds another increased layer of complexity, exploiting the resistance reductions seen in both cycling and combination treatments in order to further inhibit antibiotic resistance evolution.

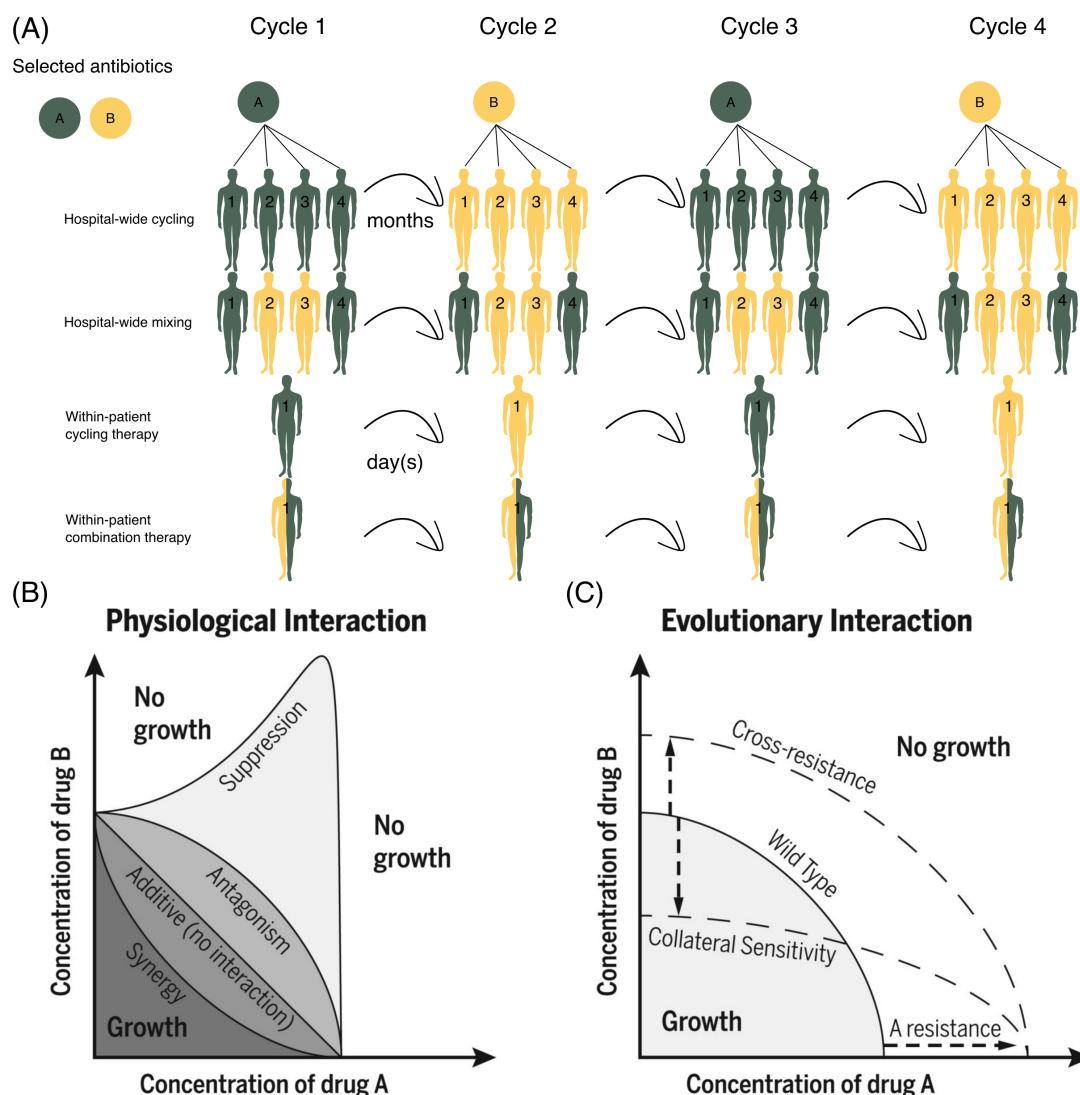


Figure 3 Multidrug strategies that aim to limit antibiotic resistance evolution. (A) Within-hospital cycling and mixing are drug stewardship strategies that aim to limit the spread of antibiotic drug resistance and involve either the cycling of drugs in a pre-specified time span or the distribution of differing drugs to patients (mixing). During drug cycling, the switch between the drugs usually occurs on a monthly basis. For example, the hospital will dispense drug A for January, and drug B in February. Drug mixing involves distributing differing drugs for patients diagnosed with similar diseases and housed in close proximity. Within-patient cycling and combination therapies aim to prevent the emergence of drug resistance, and involve either the cycling or concurrent use of multiple drugs within a single

patient. The cycles within patients would theoretically last 12-24 hours, depending on patient status and antibiotic half-life. **(B)** Physiological drug interactions can occur through simultaneous use of multiple drugs during antibiotic therapy, and the administered drugs can show additive, synergistic, antagonistic, or suppressive relationship. **(C)** Evolutionary interactions occur when bacteria evolve resistance to one drug and this resistance correspondingly translates to a hypersensitivity or resistance to a second drug. Parts B and C of this figure were made and adapted from Baym *et al.*, 2016⁵⁸.

Cycling-in-combination as a novel approach to reduce resistance evolution

As outlined above, deploying antibiotics in combinations^{214,225,226} or cyclically^{201,237,239} are strategies that have previously been shown to neutralize the advantage present in resistant bacterial populations undergoing antibiotic treatment in comparison to conventional monotherapies (Fig. 4). However, the success of these multidrug treatments is largely contingent on the evolutionary and physiological interactions present when applying multiple drugs to a single treatment protocol. Cycling drugs in combination aims to further offset the selective advantage resistant bacterial cells possess during antibiotic treatment by exploiting the already-observed advantages posed in each of the multidrug treatments

It is important to note that the goal of cycling-in-combination therapy is not to avoid the inevitable by stopping evolution, as bacteria are experts at adapting, even to the most toxic environments. The purpose of this study is rather to decipher the complex evolutionary dynamics in response to multidrug treatments, to learn how to slow down evolution, to decrease rates of multidrug resistance, and to ultimately steer evolution. By allowing antibiotics to act as shepherds of bacterial evolution, these drugs guide bacteria towards paths of resistance that can be subsequently be effectively treated by additional drug(s). I postulate that cycling drug combinations has the potential to slow down evolution of antibiotic resistance based on three main criteria: (1) environmental fluctuation, (2) increase in environmental complexity, and (3) exploitation of both physiological and evolutionary interactions present in multidrug environments.

Similar to cycling, cycling drugs in combination involves rapid switches of component drugs, thus producing rapid environmental fluctuations. This constant change translates into dynamic adaptive landscapes in which the evolution of resistance is neither easily nor quickly acquired^{234,242}. As outlined above, fluctuating antibiotic environments have been shown to decrease resistance evolution and to increase bacterial extinction^{201,237-239,243}. The success of cycling drugs has been attributed to the presence of reciprocal collateral sensitivity^{217,239} and to the presence of negative hysteresis²⁰¹. However, if the designed cycling therapy contains antibiotics that display collaterally resistant interactions, the treatment can select for multidrug resistant bacteria (Fig. 4B). It is therefore of extreme importance to carefully select the deployed antibiotics, and to further understand their component interactions.

The simultaneous application of multiple drugs increases environmental complexity by targeting several molecular sites, thus decreasing the likelihood of single step mutation generating resistance. The engagement of multiple bacterial target sites has been suggested to be responsible for the success of many particularly-effective monotherapies²⁴⁴. Deploying more than one drug in parallel extends this result, by explicitly adding drugs with diverse molecular targets, thus increasing environmental complexity. Drug combinations have been shown to slow resistance evolution^{223,225}. The physiological interactions dictate treatment success for antibiotic combinations. While synergistic combinations offer a higher bacterial extinction rate, antagonistic and suppressive drug interactions slow down bacterial resistance evolution and offer an

extended efficacy of the used drug combination^{214,222,223,225,245} (Fig. 4C). However, further experiments found no significant correlation between interaction profile and rate of adaptation, but instead observe a correlation between extinction rate and synergistic combinations¹⁹⁸. While two-member combinations have been previously explored, the addition of more drugs to a single treatment protocol has been, to my knowledge, widely ignored. However, recent evidence suggests that the frequency of antagonism and synergism increases as the number of antibiotics in the environment increases²⁴⁶. As these interactions hold the key to rational drug design, I argue that treatments involving more than two drugs are an uncharted territory that have the potential to create additional successful treatment strategies.

Cycling drug combinations incorporates a fluctuating and complex environment to create a rugged adaptive landscape that challenges bacterial adaptation. Although the encompassing physiological and evolutionary interactions of utilized antibiotics for cycling-in-combination therapy have the potential to decrease adaptation rate, the multifaceted presence of many simultaneously occurring interactions makes drug choice more complicated than for cycling or for combination treatments alone. I therefore chose to solely focus on physiological interactions when choosing antibiotics for each treatment. I hypothesized that although synergistic and antagonistic combinations both offer the potential to decrease resistance rate, drug combinations involving antagonistically interacting antibiotics could show increased capacity to decrease resistance evolution. For example, drugs A+B interact antagonistically and are applied during season 1, the population subsequently evolves resistance to drug A (as the effect of the drug B is masked by the antagonism of the pair). When the bacteria are hit with the second antagonistic antibiotic combination (drugs C+D), the bacteria not only are still sensitive to drug B from the first combination, but also ideally have sensitivity to 2 additional drugs in the rotor (Fig. 4D). Based on the above-mentioned reasons, I speculated that cycling drug combinations is an interesting, alternative treatment option that offers many ways in which the treatment can be manipulated to produce more effective protocols.

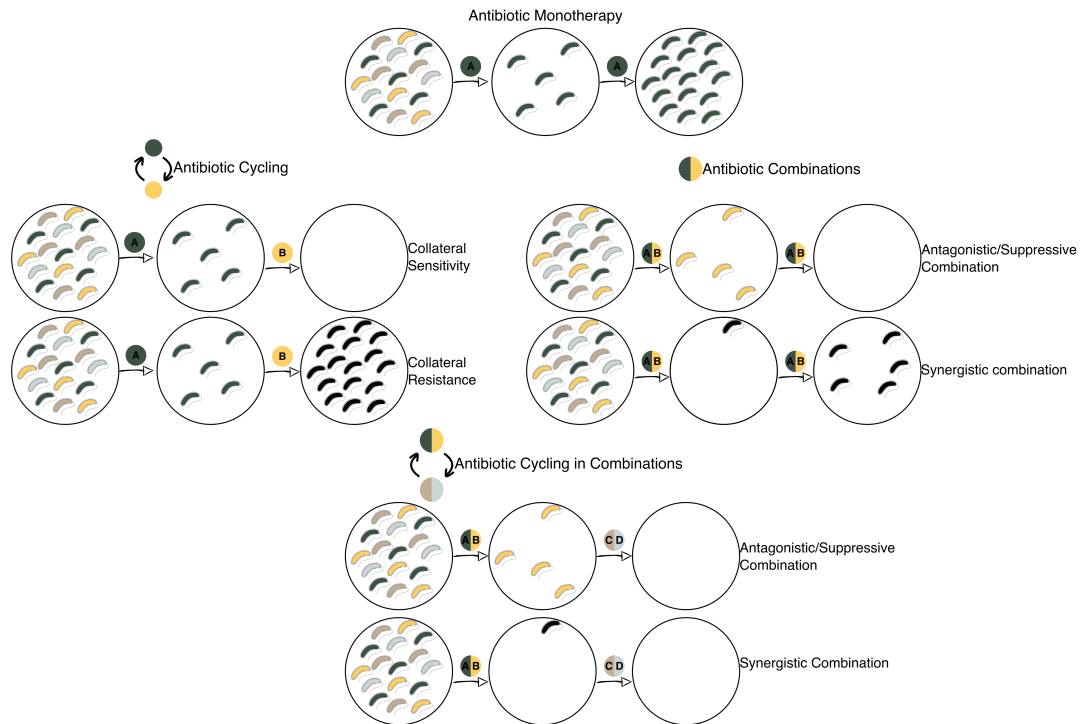


Figure 4 Steering evolution with multiple antibiotic selective pressures. Antibiotic resistance evolves as a response to the antibiotics being used as a selective pressure. Antibiotics are shown as circles, with each color representing a different drug. When a bacterial population is exposed to a drug, the drug selects for bacterial cells able to grow in said drug's presence (denoted by coloring the bacterium in the color of the antibiotic to which they are resistant) **(A)** During the conventional monotherapy, drug A selects for either the randomly present, or for the spontaneous mutator harboring the mutation necessary to grow under this static selective pressure. **(B)** During antibiotic cycling, evolutionary interactions can be exploited by allowing the population to select for or to evolve resistance against drug A. Applying drug B results in increased sensitivity relative to the ancestor to drug B, and thus a more effective treatment (collateral sensitivity) or decreased sensitivity to drug B, and thus a poorer treatment (collateral resistance) resulting in multidrug resistance (denoted as the color black). **(C)** Antibiotic combinations also provide opportunities to exploit physiological interactions. Synergistically interacting drugs produce increased extinction, but also the possibility to evolve multidrug resistance, as this high, static selective pressure can select for non-specific resistance mutations. Antagonistically- or suppressively-interacting drugs, because the effect of one drug is suppressed by the second, allow for the population to evolve resistance to one of the two drugs in the combination. This resistance, in turn, removes the suppressive effect of drug A, and the bacteria are sensitive against the drug B. **(D)** By using the physiological and evolutionary interactions present in each of the component therapies, cycling-in-combination therapy can theoretically use both synergistic and antagonistically-interacting antibiotics to successfully slow down resistance evolution. If synergistic combinations are deployed, the switch from drugs A+B to drugs C+D creates a complex, fluctuating environment, making it unlikely for multidrug resistant bacteria which are able to resist drugs A+B in combination 1 to also resist the second combination of drugs C+D. If antagonistic drug pairs are used, and the bacteria are able to evolve resistance against either drug A or B in the first combination, the fluctuating environment and the addition of two new drugs in the second combination creates an extremely difficult environment to evolve multidrug resistance.

Aim and Motivation

During my PhD, I aimed to study how combination, cycling, and cycling-in-combination therapies contribute to the long-term evolution of resistance in the clinical strain *Pseudomonas aeruginosa* PA14. The strain used in this study was isolated from a human burn wound and is highly virulent²⁴⁷. PA14 is a widely used reference strain and represents the most common clonal group of *P. aeruginosa*²⁴⁸. While *in vitro* evolution experiments have shown antibiotics used in combination^{214,225,226} and cycled^{201,217,237–239} have potential to slow down resistance evolution, the reasons behind this potential are not yet fully clear. Evolutionary and physiological interactions have been shown in many circumstances to be responsible for the reduction in the evolution of resistance⁵⁸, however the interaction-type that has the greatest impact on resistance evolution, and the interplay between the two interaction types is not yet agreed upon. Physiological and evolutionary interactions have previously been measured in our lab with our specific strain^{198,199}. I used this data to design and carry out a systematic study to optimize antibiotic cycling, as well as to compare the efficacy of cycling, combination, and cycling-in-combination regimens (Fig. 5). To test these variables, I performed a series of 7-day evolution experiments. I used an assortment of antibiotics in order to determine how the interaction of antibiotics affects evolution of resistance. I included combinations displaying both synergistic and antagonistic interactions to test for the effects these interactions have on the evolution of the populations. Ensuing evolution experiments, I characterized selected evolved material by looking for differences in overall fitness, population heterogeneity, and resistance patterns. All of this data together allows us to further understand the impact alternating drugs, cycling antibiotics in combination, and combining drugs have on the evolution of resistance in the strain PA14 of *P. aeruginosa*.

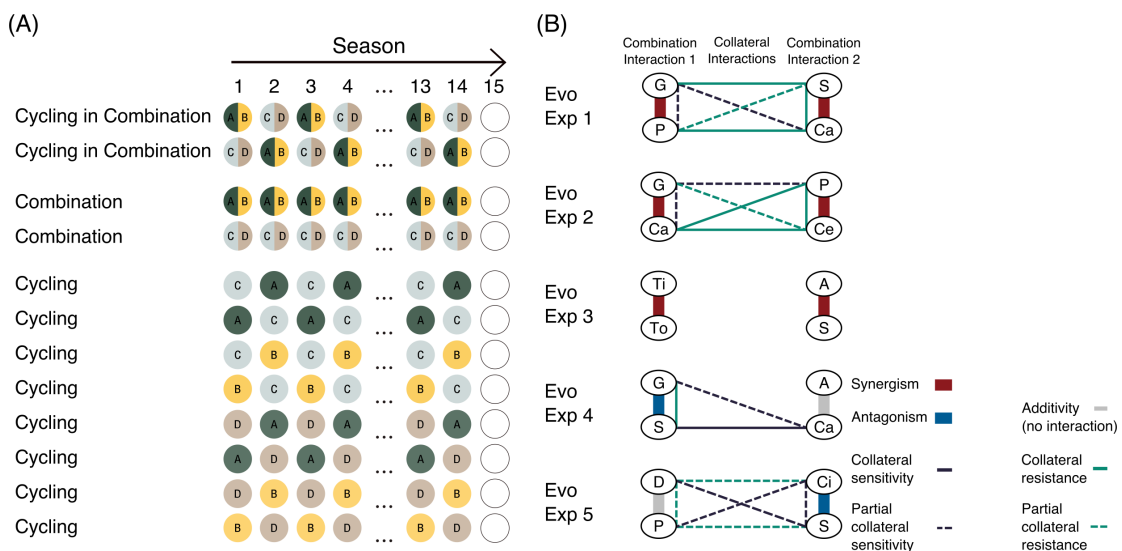


Figure 5 Evolution experimental setup and corresponding evolutionary and physiological antibiotic interactions. Each evolution experiment was designed to compare three regimen types, including cycling-in-combination, combination, and cycling. Each color and letter indicate a single antibiotic. Contained in each regimen are a specified number of treatments. Each treatment is composed of a differing sequence of component drugs (see Materials and Methods). (B) Each of the five evolution experiments contains a total of four drugs; their type of interaction and their likely evolved collateral effects are indicated, as far as available from previous work¹⁹⁸. Abbreviations for used drugs are shown in Table 2.

Table 2 Utilized antibiotics and their corresponding abbreviations, drug class, and functional targets.

Abbreviation	Drug	Class	Functional Target
D	Doripenem	Carbapenems	Cell Wall Synthesis
P	Piperacillin+ Tazobactam	Penicillins	
A	Azlocillin		
Ca	Carbenicillin		
Ti	Ticarcillin		
Ce	Cefsoludin	Cephalosporins	
S	Streptomycin	Aminoglycosides	Protein Synthesis
G	Gentamycin		
To	Tobramycin		
Ci	Ciprofloxacin	Fluoroquinolones	DNA Repair

Materials and Methods

Bacterial cultivation

All experiments were conducted with *Pseudomonas aeruginosa* strain PA14. Bacteria were generally maintained on LB agar plates. PA14 was streaked out to isolate single colonies from a frozen stock. For preparation of liquid cultures, a single colony of PA14 was inoculated in M9 minimal medium supplemented with 0.2% glucose (w/v) and 0.1% casamino acids (w/v). The culture was shaken at 37°C for approximately 6 hours, or until an optical density at 600nm wavelength (OD₆₀₀) of 0.08 was reached, corresponding to mid-exponential phase. The culture was diluted down to an OD₆₀₀ of 0.08 (if necessary), and the diluted cultures were subsequently inoculated into the experiment.

Evolution experiments

I allowed PA14 to evolve for approximately 80 generations in serial dilution experiments (Fig. 6). Using a 2% bottleneck, transfers (further called seasons) were completed every 12 hours, for a total of seven days. Experiment one lasted one season less than the remaining experiments, as a result of a malfunction of plate reading devices. The final transfer (season 15 for all experiments excepting experiment 1) was placed into drug-free media in order to test if populations showing little or no growth were extinct or simply showing a slow growing or dormant phenotype. Five independent sets of evolution experiments were performed, consisting of 10 antibiotics spanning four drug classes, with four antibiotics per experiment (Fig. 5, Table 1). All evolution experiments had a corresponding evolved control to assess the presence of a possible selective pressure other than the addition of antibiotics, and empty wells were included to control for contamination. Chosen antibiotics were selected based on their physiological interactions, as previously measured by Barbosa *et al*¹⁹⁸. The evolutionary interactions between drugs were also measured for many of the selected antibiotics in our PA14 strain¹⁹⁹, however, as these interactions are not always consistent and can show multiple paths towards evolution, I focused on the effect of physiological interactions when choosing the drugs for each experiment. Three experiments included combinations displaying synergistic interactions, and the remaining two contained at least one drug pair displaying antagonistic interactions (Fig. 5).

The main objective when designing experiments was to contrast between three types of alternative therapies: cycling-in-combination, cycling, and combination regimens. However, the cycling-in-combination regimen inherently must have four antibiotics, while cycling and combination intrinsically contain two. Therefore, in order to ensure that the observed differences were a consequence of the regimen and not the specific antibiotics being applied, I included the four antibiotics used in the respective cycling-in-combination regimen in both the cycling and combination regimens. This resulted in a total of 12 treatments, with several treatments belonging to each regimen (Fig. 5). The combination regimen contained two treatments, each with one of the two drug pairs used in the respective cycling-in-combination regimen. The cycling regimen contained a total of eight different treatments, to control for each switch present in the respective cycling-in-combination regimen. For example, a specific cycling-in-combination treatment may be composed of antibiotics G, P, S, Ca. Season one contained drugs G+P, and season 2 transitioned to antibiotics S+Ca. The corresponding cycling treatments would contain a treatment cycling from G to either S or Ca, and

additionally from P to either S or Ca, for comparability between the two regimens. Additionally, the order in which antibiotics are deployed has been shown to have an effect on evolutionary outcomes^{201,237,249}. To control for order effects, regimens with fluctuating environments contained additional treatments that included the reverse order of each treatment sequence. For example, when cycling-in-combination treatment one cycled from antibiotics G+P in season one to S+Ca in season two, treatment two contained drugs S+Ca in season one and switched to G+P in season two. The same was performed for each pair of drugs used in the cycling treatment. Every treatment was replicated a total of 8-10 times and spread out over 2-3 96-well plates using a randomized block design to control for gradient effects.

An inhibition concentration of 80 (IC₈₀, +/- 10%) was standardized for each treatment prior to the start of the evolution experiment. This concentration was chosen in order to exert a substantial selective pressure that also has clinical relevance on the bacteria, while also allowing evolution to take place. Antibiotic tissue penetration varies and does not always achieve 100% of the desired drug concentration in all areas of the body²⁵⁰⁻²⁵², thus IC₈₀ values may also be seen in select *in vivo* bacterial infections. At the beginning of each experiment, bacteria were diluted to approximately 10⁵ cells per well from a culture in mid-exponential phase. 96-well plates were prepared prior to the dilution and inoculation of PA14. The plates were then incubated shaking at 37°C in Tecan plate readers. Throughout evolution, OD₆₀₀ was measured continuously (every 15 minutes) in order to accurately track and follow evolutionary dynamics. Following 12-hour incubation, the plates were removed from the machines and a bottleneck volume of 2% was transferred to the subsequent plate with fresh media and the corresponding antibiotic required by the treatment type. Bacteria from evolution experiments were frozen every second transfer in 25% glycerol, and then stored at -80°C for future analysis.

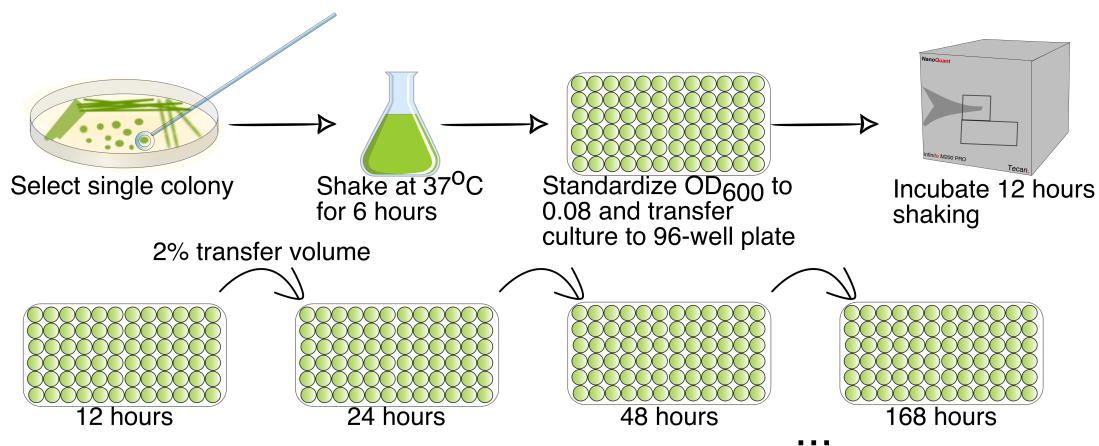


Figure 6 Evolution experimental setup. Evolution experiments were performed via classic serial transfer. A single colony was randomly selected from a previously streaked LB agar plate, inoculated into M9 minimal media, and incubated shaking at 37°C until reaching exponential phase. During incubation, 96-well plates were prepared containing the corresponding antibiotic treatments. After roughly 6 hours of incubation, OD₆₀₀ of the shaking culture was measured, and diluted (if necessary) to 0.08, corresponding to mid-exponential phase. The culture was then inoculated into the 96-well plate containing unique antibiotic treatments, incubated for 12 hours, and 2% was continually transferred to the subsequent plate for a total of 168 hours.

Dose response curves

As the goal of our evolution experiments was to compare between different antibiotic regimens, each containing differing drugs, the inhibition concentration had to be standardized for each bacterial population prior to the evolution experiment. This standard inhibition of 80% for all bacterial populations allows the direct comparison of the effect of regimens on bacteria adaptation, as the initial selective pressure exerted by the drugs will be approximately equal. Dose response curves were performed to gain an understanding of how the bacteria responded to single or multidrug environments (Fig. S1). For single drug environments, bacteria were grown, and transferred to increasing concentrations of a single antibiotic (n=8). After 12 hours of incubation, shaking at 37°C in Tecan plate readers, the OD₆₀₀ of the bacterial culture was measured. A period of 12 hours was chosen, as incubating the bacteria for increased time (24 hours) showed a substantial increase in variation of bacterial inhibition, making standardization of the inhibition concentration for the beginning of the evolution experiment impossible (Fig. 8). These measurements were analyzed using R statistics software²⁵³, using the “drc” package²⁵⁴, which uses a logistic regression model to estimate the concentration of antibiotic in which 80% of the bacteria are inhibited. Multidrug environment inhibition was measured by first measuring the dose response curve of each of the single drugs in the combination. The concentration at which 50% of the bacteria were inhibited (IC₅₀) was then taken for each of the component drugs and placed in a 2 ml stock solution. Serial dilutions were made from the stock solution to perform the subsequent dose response curve containing two drugs, and the concentration in which 80% of the bacteria were inhibited was identified in order to start the evolution experiment.

Statistical analysis of growth dynamics, extinction, and growth effects

All analyses following the evolution experiment were carried out using R statistics software²⁵³. Area under the curve (AUC) was calculated using the ‘MESS’ package in R²⁵⁵ for each replicate population at each transfer for all evolution experiments in order to compare growth of the bacteria under different selective regimens. AUC is a compound measurement that incorporates lag time, exponential growth, and carrying capacity of the microbial population, thus creating a more compound measurement to monitor bacterial evolution over time, and has been found to be an informative parameter to track evolutionary dynamics^{222,237,239}. Since extinct populations drastically influence AUC, I removed non-growing populations from these analyses. Instead, extinction was used and analyzed as a separate phenotype for assessing treatment efficacy. Statistical analysis on cumulative AUC data, averaged for each regimen, was performed using the ‘lme4’ package in R²⁵⁶. Each individual evolution experiment was analyzed separately using a linear mixed-effects model, where AUC was the response variable, treatment was the fixed factor, and replicate number and season were random factors. Significance of differences between regimens were subsequently assessed using the R package ‘multcomp’²⁵⁷ by performing a Tukey’s honest significance difference test (HSD), and *P*-values were adjusted to control false discovery rates (fdr) (Table S2). In addition to cumulative AUC, AUC inhibition (AUC_{*i*}) plotted over time allowed for the visualization of adaptation dynamics throughout evolution. AUC_{*i*} is a relative measurement that standardizes inhibition compared to the evolved control, calculated as:

$$AUC_i = \frac{1 - AUC_{treatment}}{AUC_{evolved\ control}}$$

Extinction rates were calculated by first qualifying whether a replicate population was, in fact, extinct. Each replicate population was separately analyzed and classified as extinct if growth during the final, drug-free control season was equal to or under a threshold value. The threshold value was determined by the empty control well, which contained only media and no bacterial cells. Extinction rates were calculated by dividing the number of extinct wells by the total number of replicates per regimen. Extinction was analyzed statistically by performing a generalized linear model (GLM) using the R package ‘glm2’²⁵⁸. Each model was performed with a binomial distribution, where extinction was the response variable, and regimen the explanatory variable. An HSD was performed following the GLM, and *P*-values were adjusted by *fdr* (Table S3).

Fitness effects were assessed by analyzing growth curves of bacterial populations during the final season in drug-free media. The different parameters of growth (i.e., lag time, growth rate, maximum yield) were measured in R using the package ‘grofit’²⁵⁹. The relative values of each of these parameters were calculated using the mean of the corresponding evolved control populations, simultaneously evolved in no-drug media, as a reference (n=8) using the following equation:

$$\text{Relative maximum yield}_{\text{regimen1}} = \frac{\text{Mean maximum yield}_{\text{regimen1}} - \text{Mean maximum yield}_{\text{evolved control}}}{\text{Mean maximum yield}_{\text{evolved control}}}$$

Statistical tests on this data were performed using a linear mixed-effects model with the considered growth parameter averaged for each regimen as the response variable, regimen as the fixed factor, and row and plate as random factors. Significance of differences was evaluated via HSD and corrected by *fdr* (Table S4, S5, S6).

As both physiological^{217,239} and evolutionary^{198,214} interactions between applied drugs have been shown to influence treatment success, associations between treatment efficacy (measured by cumulative AUC and extinction rate) and drug interactions were performed. Both physiological and evolutionary drug interactions were previously measured in PA14 by Barbosa *et al*^{198,199}. In short, frequency of collateral resistance (FCR)¹⁹⁹ was used to report evolutionary interactions, with numbers ranging from 0 to 1. An FCR score of 0 indicates that all tested populations displayed collateral sensitivity to the utilized drugs, while a score of 1 reports all tested populations exhibited collateral resistance to the second drug. Physiological interaction scores (PIS) were used to test for the presence of drug synergism or antagonism, and scores ranged from -0.4 to 0.2. A PIS score of -0.4 illustrates strong antagonism and 0.2 indicates strong synergism between utilized drugs¹⁹⁸. The impact of both FCR score and PIS were separately analyzed for combination and for cycling regimens. First, an association of physiological and evolutionary drug interactions on extinction rate was analyzed using a GLM with a binomial distribution using the ‘lme4’ package (n = 8-12 replicates per treatment, depending on experiment, n = 2 treatments per experiment) (Table S13, Fig. S3-S4)²⁵⁶. Presence or absence of extinction for each replicate was the response variable, either PIS or FCR scores was the explanatory variable, and antibiotics used (treatment) and experiment were random factors (Table S13). Second, a linear mixed effects model assessed the relationship between drug interactions (PIS or FCR) and cumulative AUC (n=8-12, depending on experiment and extinction rate, n = 3-8 treatments per experiment, depending on the availability of FCR scores), and was performed using the ‘lme4’ package (Table S13, Fig. S3-S4)²⁵⁶. Following the model, Wald tests were run using the ‘car’ package to attain *P*-values²⁶⁰. The presence of a significant association was also tested for drug interaction (PIS or FCR) and fitness costs (measured by relative maximum yield) for combination and cycling treatments

individually. As an ANOVA between the null and the desired model did not show that the desired model to be run accounted for more of the variance than the null model, analyses via a linear mixed-effects model was not continued. No significant association between any drug interaction and fitness cost was identified for combination nor for cycling regimens (Table S14, Fig. S7).

Characterization of evolved material

Characterization of evolved material provided resistance profiles to the tested antibiotics, as well as to identified population heterogeneity (Fig. 7). Evolution experiment 5 was chosen, based on its extreme variation in inhibition during opposing seasons (strong zigzag dynamics), indicating presence of subpopulations with differing sensitivities to component drugs (Fig. 14E, Fig. S8). The populations were tested from two time points of the frozen evolution experiment (season 7 and season 14) to contrast the effect of increased antibiotic exposure and evolutionary time on resistance. 8 of the 12 treatments were tested (Table S1) and were chosen based on the level of zigzag dynamics the population showed during evolution (Fig. 14E, Fig. S8). From each treatment in the evolution experiment, three of the eight replicates were tested (Fig. S8). The wells that were chosen showed contrasting inhibition levels to check for different evolutionary paths of resistance. During subpopulation experiments, frozen 96-well plates were removed from -80°C , foil was detached, and plates were immediately transferred to 37°C shaking for 5-10 minutes, until all wells were fully thawed. 50 μl from the desired wells was then pipetted into 450 μl PBS solution in order to subject the stressed cells to an isotonic environment. Serial dilutions were subsequently performed, and 100 μl from the serial dilutions was then plated on LB agar plates and incubated for 24 hours, or until colonies of appropriate size were grown. Following LB plate incubation, 20 colonies from a single population were chosen and inoculated into separate falcon tubes, each containing 2 ml of M9 minimal media, and shaken for 6-8 hours until an OD_{600} of 0.08 was reached. The colonies were then subjected to increasing concentrations (8-10 concentrations in total, spanning from IC_{10} to 16x MIC) of each antibiotic received during treatment in order to identify phenotypic differences in resistance. The 96-well plates were prepared during the incubation, and when cultures reached sufficient density, 10 μl from each culture was inoculated into each well of a 96-well plate containing varying concentrations of the component antibiotics of the particular treatment. The plates were then incubated for 12 hours at 37°C shaking, and were read after 12 hours of incubation on Tecan plate readers.

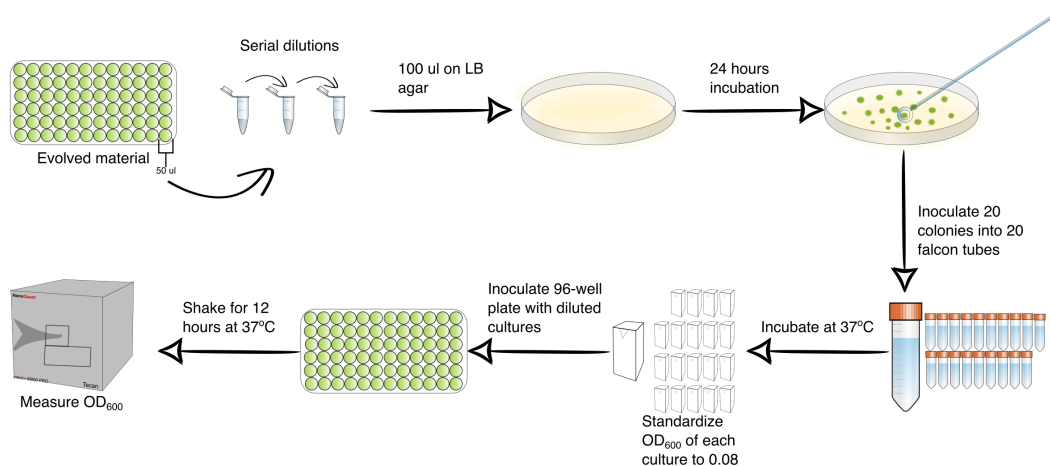


Figure 7 Evolved material characterization. Evolved material was removed from -80°C and thawed at 37°C shaking for roughly 1 minute. $50\ \mu\text{l}$ from each chosen well was subsequently removed, and serially diluted and plated on LB agar plates. Plates that contained a minimum of 20, easily differentiable colonies were chosen, and 20 colonies from a single plate were subsequently inoculated into 20 falcon tubes containing M9 minimal media. The falcon tubes were incubated, shaking at 37°C for approximately 8 hours, or until an OD_{600} of 0.08 was achieved, corresponding to mid-exponential phase. The cultures were then subjected to increasing concentrations of the antibiotics, and further incubated for 12 hours. OD_{600} was measured after 12 hours to gain understanding as to how select antibiotic treatments affect evolution of resistance.

Statistical analysis of evolved material characterization

All analyses following subpopulation assays were carried out using R statistics software²⁵³. Hierarchical clustering was performed in order to identify heterogeneity within single bacterial populations. The core R package ‘stats’ was used for all clustering. Clustering was performed for each bacterial population (each well in the evolution experiment, which contained a total of 20 measured colonies). Each colony contained OD_{600} measurements for 8-10 increasing concentrations of the component antibiotics seen in the different treatments, all of which were included in the clustering analysis. The first two concentrations of antibiotic were removed, as these concentrations were very low inhibition values, and showed too much variation not attributed to resistance. OD_{600} measurements were then compared to the PA14 control used during each subpopulation experiment, and the change in resistance per concentration was calculated from these values as:

$$\begin{aligned} \text{Change in Resistance}_{\text{concentration 1}} &= \text{OD}_{600 [\text{concentration 1}] \text{ of PA14 control}} \\ &- \text{OD}_{600 [\text{concentration 1}] \text{ of specified colony.}} \end{aligned}$$

The changes in resistance for each of the concentrations were subsequently summed to obtain the total change in resistance over the range of concentrations. These measurements represent the level of resistance the colonies have evolved when compared to their ancestor strain. Euclidian distance was used to measure change in resistance distances between colonies belonging to one population, and clustering was performed using the Ward’s minimum variance method²⁶¹. Each population was tested against the antibiotics seen during evolution, clustering was performed using information derived from all of the tested antibiotics, and the maximum number of clusters observed per antibiotic was taken as the final cluster number. In order to avoid biases based on the number of antibiotics tested, colonies belonging to the cycling-in-

combination regimen were also clustered based on 2 out of the 4 tested antibiotics. 2 antibiotics were randomly chosen using the R command “sample”, and this simulation was replicated a total of 100 times in order to avoid biases based on the number of included antibiotics. Clustering was then performed on each of these 100 trials, and the median number of clusters was taken from this data set and included as the final number of clusters. The dendrograms were cut at a height that corresponded a single cluster in control, evolved populations. These wells controlled for normal variations as well as any selective effects the evolution experiment might have imposed in addition to the antibiotic pressure.

Differences in the change in resistance from season 7 to season 14 were assessed per isolated colony and per tested antibiotic. Significant differences between seasons were investigated by performing a mixed linear model where change in resistance per antibiotic was the response variable, season was the fixed factor, and regimen was a random factor. HSD was used to evaluate significance of differences and *P*-values were corrected to control for false discovery rate (Table S10).

Shannon’s diversity index was calculated in order to compare population heterogeneity in a single well and between regimen types. Calculations were performed via the ‘vegan’ package²⁶². Following calculations, a linear model was performed on the data to assess significant differences between regimens. The model considered the Shannon diversity index as the response variable, regimen as the fixed factor, and transfer as a random factor. Significance of differences was evaluated via HSD and corrected to control for false discovery rate (Table S15).

Multidrug resistance (MDR) indices were calculated to test if a single regimen increased prevalence of resistance to more than one of the utilized drugs. The changes in resistance per drug were added and divided by the total number of drugs in the treatment. Since cycling drug combinations contained 4 drugs, 2 antibiotics out of the 4 tested drugs were randomly chosen using the R command “sample”. This was performed 100 times, and the mean MDR index of all the trials performed was taken for the analyses. Differences in MDR indices were subsequently tested for significant differences between regimens by performing a mixed linear model where multi-drug resistance index was the response variable, regimen was the fixed factor, and transfer was a random factor. HSD was used to evaluate significance of differences and *P*-values were corrected by *fdr* (Table S9).

Fitness costs of characterized evolved material

To further investigate associated fitness costs associated with the attained resistance phenotypes, additional fitness cost assays were performed. The frozen glycerol stocks from transfer 14 subpopulations containing corresponding colony numbers were streaked on LB agar and incubated overnight at 37°C. One colony was chosen from the resulting LB plate, and inoculated into 2 ml of M9 minimal media for 6 hours at 37°C while shaking. The resulting culture was diluted to an OD₆₀₀ of 0.08, corresponding to mid-exponential phase. 10 µl from the diluted culture was pipetted into 90 µl of M9 minimal media in 96-well plates and shaken at 37°C for 16 hours (n=3). This phase of pre-adaptation was performed in order to adapt the culture from freezing to liquid media conditions and to ensure that any observed fitness effects were indeed due to the evolved resistance and not simply to the shock from frozen to liquid media. Following pre-adaptation, 10 µl from each well was transferred directly into 90 µl of fresh M9 media, and 24 hours of incubation at 37°C was performed in Tecan plate readers.

During incubation, plates were continuously shaken and OD₆₀₀ was measured every 15 minutes.

Following measurements, fitness parameters were again calculated using the 'grofit' package in R statistical software²⁵⁹. Relative fitness measurements were calculated using the PA14 ancestor strain via the following equation:

$$\begin{aligned} & \textit{Relative maximum yield}_{\textit{colony 1}} \\ &= \frac{\textit{Calculated maximum yield}_{\textit{colony1}} - \textit{Mean maximum yield}_{\textit{PA14 ancestor}}}{\textit{Mean maximum yield}_{\textit{PA14 ancestor}}} \end{aligned}$$

Results

To address how differential drug application affects evolutionary dynamics, I performed a series of 5 *in vitro* evolution experiments. The main research goal was to compare how differing drug regimens, involving the same set of antibiotics, deployed both temporally and spatially differentially, affected treatment efficacy. Treatment efficacy was defined by measuring four bacterial phenotypes throughout and following evolution: extinction rate, growth inhibition, fitness costs, and presence of single and multidrug resistance. Each of the chosen phenotypes provides distinctive information on how bacterial populations cope and respond to diverse environments. Evolution experiments included an arsenal of 10 drugs belonging to 5 differing drug classes (Table 2). Each experiment setup consisted of 4 antibiotics distributed in 3 different drug regimens (cycling, combination, and cycling-in-combination), and each regimen contained several treatments to control for drug-specific and drug-order effects (Fig. 5). Evolved material from chosen treatments was subsequently characterized to monitor resistance patterns and population heterogeneity.

24-hour seasons result in large inhibition variations

In order to ensure the direct comparison of regimens, each bacterial population brought into the evolution experiment was initially inhibited to the same extent. Dose response curves were thus performed prior to the evolution experiment to acquire the precise antibiotic concentrations needed to initially inhibit a bacterial population by 80% relative to bacterial growth in no-drug media (inhibition concentration of 80%, further referred to as IC_{80}). Previous colleagues chose to observe evolutionary dynamics in 12-hour intervals (further referred to as seasons), meaning that the drugs were deployed and incubated along with bacteria for 12 hours, bacteria were transferred at a pre-chosen passage rate, and subsequently subjected to a new, fresh round of drugs^{198,199,201,237}. I aimed to extend the season length, and to investigate evolutionary dynamics of bacteria exposed to antibiotic treatments for 24 hours. The selection of longer incubation times was chosen for three main reasons. First, many drugs are given to patients only once a day, thus the dynamics of once-a-day cycling treatments were relevant, and, to my knowledge, had not yet been studied in PA. Additionally, a recent meta-analysis found that patients were generally more likely to take their drug in a timely manner when given once-a-day treatments when compared to drugs being taken 2-3 times daily, increasing treatment efficacy due to adherence to treatment schedule²⁶³. Secondly, the extension of the season length would also correspond to an increased time in stationary phase. I found this extended stationary phase relevant, as PA is naturally a soil-dwelling bacterium, and environmental microorganisms are rarely found in exponential phase, but spend the majority of their lifetime in stationary phase²⁶⁴. Infective biofilms also often contain metabolically inactive cells which results in increased antibiotic tolerance²⁶⁵, and environments like the cystic fibrosis lung contain oxygen-deficient microenvironments which also select for slow bacterial growth^{266,267}. This extension of stationary phase would allow the study and comparison of increased time spent in stationary phase on bacterial evolutionary dynamics. Finally, increased time in stationary phase has also been found to increase the presence of persister cells both *in vivo*²⁶⁸ and *in vitro*²⁶⁴, and the presence of persister cells has been suggested to play a role in difficult to treat, recurrent PA infections²⁶⁹. For these reasons, I aimed to perform experiments using 24-hour season lengths.

I first aimed to generate accurate dose response curves with PA for the 24-hour season length, in order to determine the IC_{80} for each drug or drug pair. An inhibition of 80% was chosen, as this concentration is high enough to compare to clinical dosages,

as penetrance to select antibiotics in select tissues²⁷⁰ and biofilms²⁷¹ is not always 100%, but importantly this dose is also low enough to allow analysis of bacterial evolution. Dose response curves aim to attain low variation between replicates, and stepwise inhibition of the bacteria, meaning that the curve simply does not suddenly drop from low to high inhibition, but instead shows small decreases in the OD₆₀₀ corresponding to the increased antibiotic concentration.

Dose response curves showed that 24-hour season length increased the amount of drug needed to inhibit the bacterial population 80% when compared to 12-hour incubation times (Fig. 8A, Fig. S1). To further test this observation, IC₅₀ values from five independently performed dose response curves were measured from both 12 and 24-hour season length. Bacteria consistently required a higher drug concentration to achieve 50% population inhibition when the season length is extended to 24 hours (Fig. 8B). However, the magnitude of this trend depended on the utilized antibiotic. For example, gentamicin had significantly higher IC₅₀ values at 24 hours when compared to 12-hour values ($W=1.5$, P -value= 0.01, $n=5$). However, the remaining three drugs did not display statistically significant differences in mean IC₅₀ values from 12 and 24-hour seasons, likely resulting from the high amount of variance in measured IC₅₀ values at 24 hours (Fig. 8B, Table S8). Replicates additionally showed large daily fluctuations in growth inhibition following 24-hour incubation (Fig. 8A, B). This increase in variation made the standardization of inhibition values prior to evolution impossible, as the daily fluctuating amount of drug needed to inhibit the cultures would not allow me to unanimously achieve 80% inhibition across all treatments.

As the increased season length corresponded to increased incubation in the 96-well plate readers, I postulated that gradient effects present from the extension of incubation could be responsible for the high level of variation in inhibition. To investigate whether such technical artifacts were indeed the underlying mechanism of the large variations observed during 24-hour seasons, all 3 utilized plate readers were subsequently tested for gradient effects and their interference with accurate inhibition values. A single concentration (\sim IC₂₀) of each of the 4 utilized antibiotics was applied to each well in a 96-well plate, all containing the same bacterial inoculum, and incubated for 24-hours. The presence of slight gradients was found when including aminoglycoside antibiotics (gentamicin and streptomycin), corresponding to artificially high OD₆₀₀ values on the bottom right hand of the plate (Fig. 8C). Each of the 3 tested plate readers showed similar gradient patterns with inclusion of aminoglycoside antibiotics (Fig. S2), and no detectable gradients were present during incubation with penicillins. The gradient problem was solved by including block treatment randomization, and by restricting treatments containing aminoglycoside antibiotics only to the top left quadrant of the 96-well plate.

Repetition of dose response curves with 24-hour season length and with gradient correction again yielded large daily fluctuations in the amount of drug needed to inhibit the population by 80%. It was thus determined that the gradient was not solely responsible for the variation. After repeating dose response curves in 12-hour time spans (Fig. S1), I established that although 24-hour season length is relevant, the inability to standardize inhibition concentrations at the beginning of evolution would not allow me to answer my main, larger question. I therefore continued the evolution experiments using a season length of 12-hours, which allows the use of reliable inhibition concentrations when starting evolution.

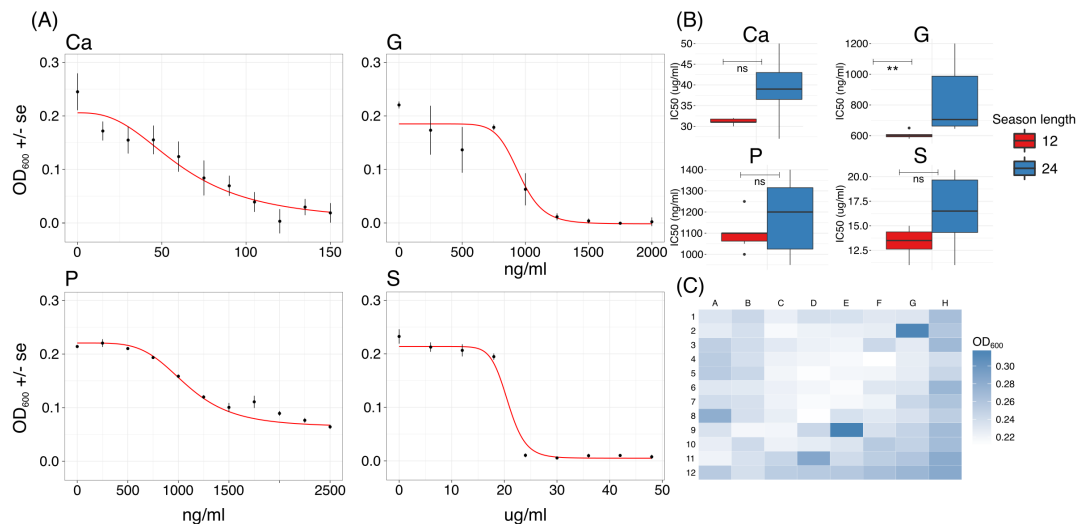


Figure 8 24-hour season length delivers increased variation in inhibition concentrations. (A) Dose response curves were performed for four chosen antibiotics, using 24-hour incubation times (n = 8-10). Abbreviations for used drugs are shown in Table 2. (B) IC₅₀ values from separately performed, independent dose response curves were calculated for 12 and 24-hour season lengths. A Wilcoxon rank sum test was performed to compare the mean IC₅₀ values for the two season lengths (Table S8, n = 5). (C) IC₂₀ concentrations were added to every well of a 96-well plate and incubated for 24 hours in the Tecan plate readers to investigate the effects of gradients of antibiotic efficacy. The above grid represents a 96-well plate with the intensity of blue color of the squares corresponding to the OD₆₀₀ measured after 24 hours incubation

Cycling antibiotics in combination leads to increased extinction rates

A series of 5 independent evolution experiments were subsequently performed using 12-hour season lengths with 10 antibiotics (Table 2) deployed differentially (Fig. 5). Following evolution, extinction rates among bacterial replicates were first analyzed to determine the influence of the antibiotic regimens on bacterial evolution. Extinction rates are a vital component in understanding antibiotic therapy efficacies, as the overarching goal of treatment in patients is to eradicate the underlying infection as quickly and efficiently as possible. In our analyses, extinction is used as proxy for selective pressure, and an increase in extinction rate is perceived as the inability of bacteria to adapt to a correspondingly high selective landscape. Each treatment in the evolution experiment contained 8-10 replicate populations, and a population was classified as extinct when no detectable growth was measured in the last season of evolution containing no drug media.

Bacterial extinction was detected in all antibiotic regimens and for each evolution experiment, demonstrating that each tested antibiotic regimen generated a sufficiently high selective pressure to eradicate select populations (Fig. 9). Cycling drugs in combination consistently produced high rates of extinction, with rates ranging from 20-70% depending on the experiment (Fig. 9). When compared to combination and cycling regimens, cycling drugs in combination yielded higher extinction rates than both of the tested alternative drug therapies, with one exception (Fig. 9C). To focus on the fluctuating environments, in 4 experiments, cycling drugs in combination yielded higher extinction rates compared to cycling single drugs (Fig. 9A, B, D, E). 2 out of the 5 experiments displayed significant differences in extinction rates between the two fluctuating regimens (Fig. 9A, B), suggesting that cycling drug combinations during these experiments created a significantly higher selective pressure for replicates undergoing evolution. Contrastingly, the remaining three evolution experiments exhibited similar extinction rates for cycling single drugs and cycling drug

combinations. These results indicate that simply adding the combination element to fluctuating environments was not always adequate to achieve higher extinction rates, and the individual component drugs may also influence extinction rates.

Static combination environments elicited the least amount of extinction in 3 out of the 5 evolution experiments (Fig. 9A, D, E). Nevertheless, only 2 experiments yielded stark differences extinction rates between combination and cycling therapy (Fig. 9D, E). In comparison to cycling drugs in combination, combination therapies presented less extinction in 4 of the 5 evolution experiments (Fig. 9A,B,D,E). However, in 2 experiments, antibiotic combinations achieved similar extinction rates to cycling drugs in combination (Fig. 9B, C), suggesting that the addition of environmental fluctuation and another set of drugs does not always equate to a significant increase in killing capacity. Notably, the remaining 3 experiments showed large differences in extinction rates for combination and cycling-in-combination environments. This finding implies that the added fluctuation and additional drugs can, in some, but not all circumstances, increase the selective pressure, and consequently decrease the probability of bacterial adaptation to these dynamic environments.

Interestingly, the experiments including one combination displaying an antagonistic interaction were interestingly the only experiments in which the combination regimens elicited no extinction (Fig. 9D, E). To test the effect of drug interaction on extinction rate, the physiological interaction scores (PIS) and evolutionary interactions (measured by frequency of collateral resistance (FCR)) for our ancestor strain of PA14 were derived from previous work^{198,199}. Tested combinations had physiological interaction scores ranging from -0.4 (strong antagonism), 0.0 (additivity) to 0.2 (strong synergism). FCR scores ranged from 0 to 1, with a score of 1 indicating all tested populations displayed collateral resistance to the second drug in the sequence, and a score of 0 indicating all populations displayed collateral sensitivity to the second drug. A generalized mixed linear model was performed using the 10 combination treatments from the 5 evolution experiments (see Methods, Table S13, Fig. S3A, C). However, the model did not find a significant association between neither FCR nor PIS on extinction rate for drug combinations, implying that additional factors other than drug interaction were responsible for the extremely low extinction rate in these experiments.

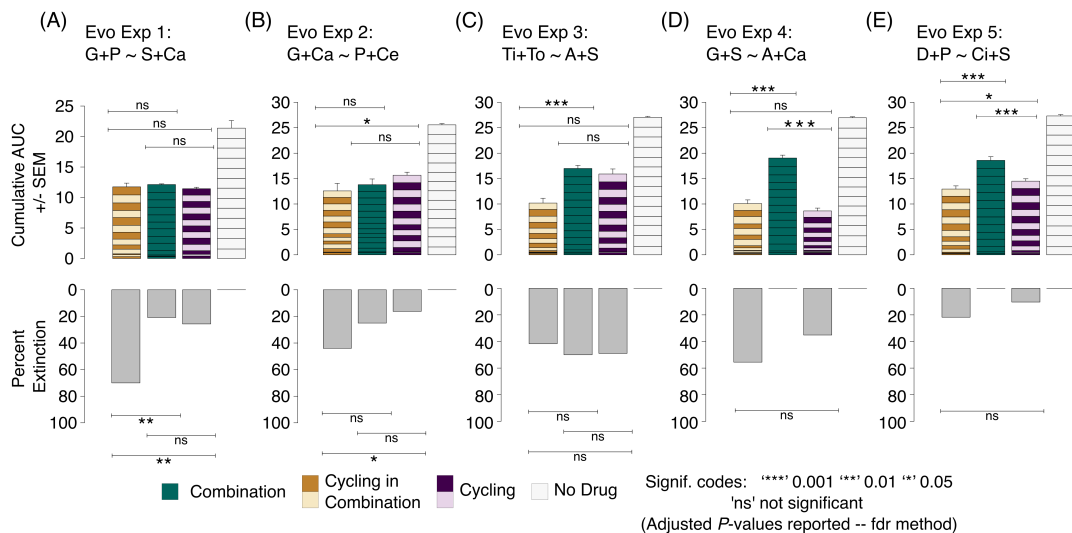


Figure 9 Growth and extinction dynamics during evolution. (A-E) Each vertically stacked box in the column represents cumulative AUC of the specified regimen per transfer. The cumulative AUC (see Methods) is first taken

for each replicate (n=8-10), averaged per treatment, and again for the total amount of treatments belonging to a single regimen (n=2-8). Error bars represent standard error of the mean (SEM). Significance values were calculated by first performing a mixed linear model, and differences between regimens were subsequently assessed by Tukey's honest significance difference test (HSD) (see Methods). *P*-values were corrected for multiple comparisons by false discovery rate (fdr) and are shown in Table S2. The proportion of extinctions is shown on the lower axes and is the total number of extinct replicates belonging to a specified regimen, divided by the total number of replicates per regimen, and multiplied by 100 to gain a percentage. Significances were calculated by first performing a GLM, then an HSD to compare means between regimens, and *P*-values are corrected by fdr, and are shown in Table S3.

Cycling antibiotics in combination yields prolonged growth inhibition

Next, the ability of drug therapies to limit bacterial growth throughout evolution was next analyzed in order to further understand evolutionary outcomes. Extinction was explored separately from growth inhibition, as these are two distinct parameters that provide different information about therapy efficacies. While extinction correlates inversely with the adaptive potential of resistance evolution, growth inhibition informs how the therapy fares in terms of limiting the surviving bacteria in the longer term. Extinct lineages were therefore removed from growth inhibition measurements, and the focus was solely put on how the surviving populations responded to specified antibiotic treatments. To evaluate growth inhibition, the cumulative area under the curve (AUC) was used as a compound measure that takes into account all phases of bacterial growth, giving a more informative parameter (see Materials and Methods).

Cycling drugs in combination generally inhibited growth most effectively, displaying the highest overall inhibition throughout evolution in 3 out of 5 evolution experiments (Fig. 9B, C, E). However, the difference in growth inhibition displayed by each of the three antibiotic regimens was not always significant. For example, when comparing the two fluctuating environments, cycling single drugs only showed significantly lower growth inhibition compared to cycling drug combinations in 2 out of 5 experiments (Fig. 9B, E), leaving the majority of the evolution experiments revealing similar growth inhibition for both cycling environments (Fig. 9A, C, D). This result suggests that although adding additional drugs to the fluctuating regimen can, in some cases, increase growth inhibition, the additional drugs are not always sufficient to elicit profound differences in growth inhibition when compared to cycling single drugs.

Similar to the extinction data, drug combinations also yielded the weakest growth inhibition in 3 evolution experiments (Fig. 9C, D, E). Cycling drug combinations generally outperformed static combination environments by showing significantly more growth inhibition in 3 out of 5 evolution experiments (Fig. 9C, D, E). This result demonstrates that adding the additional drug pair and a fluctuating environment can create a significantly more difficult adaptive landscape to which bacterial inhibition is extended. Conversely, 2 evolution experiments produced similar growth inhibition for drug combinations compared to the two fluctuating regimens (Fig. 9A, B), indicating that regimen may not be the only variable dictating growth inhibition during evolution.

Cumulative AUC provides insights to the overall growth inhibition of the bacterial population through evolution, but is unable to resolve evolutionary dynamics during the experiment. To further explore how each antibiotic therapy affected bacterial growth during evolution, the OD₆₀₀ measurements continuously collected every 15 minutes were plotted to monitor bacterial evolutionary dynamics. The mean AUC inhibition percentage was calculated, which standardizes the AUC curve relative to the

curve of the ancestor evolved in no-drug media, to attain a final percent inhibition seen by each regimen (AUC inhibition, further referred to as AUC_i). In each regimen, a sharp initial increase in inhibition was present during seasons 2-3 (Fig. 10), implying that selective pressure was the highest during these seasons, and thus the majority of sensitive bacterial cells unable to withstand the selective pressure and were eliminated. Following the high inhibition period was a decrease in growth inhibition, generally seen during seasons 3-6. This phase represents the selection of the cells capable of withstanding the exposed environment being able to grow to higher carrying capacities. However, the extent of the decrease in growth inhibition as well as the duration of the decrease differed across evolution experiments and the tested regimens (Fig. 10). After 15 seasons of evolution, bacteria that had not been eliminated from the initial high selective pressure generally grew only ~20% less than the ancestor growing in no drug media. This finding exemplifies the evolvability of PA, being that if the cells were capable of temporarily withstanding this environment, they were eventually able to grow proficiently, regardless of the drug regimen.

As observed in the growth inhibition and extinction data, combinations generally showed the fastest adaptation to the environment, with the AUC_i curve dropping substantially in seasons 3-5 (Fig. 10D, E). However, two experiments showed the combination curves intertwining with the curves of the remaining regimens, again signifying that drug regimen was not the only predictor of adaptation rates (Fig. 10A, B). Cycling drugs in combination appeared to be the most difficult adaptive landscape for the bacteria, as the inhibition of this regimen generally remained high until season 7-9. Two experiments displayed the cycling-in-combination growth inhibition curve well above the remaining regimens, suggesting that this regimen prevents bacterial growth for an extended period of time compared to the alternative regimens (Fig. 10C, E). Nonetheless, three remaining evolution experiments showed populations undergoing cycling-in-combination therapy producing evolutionary dynamics similar to cycling single drugs (Fig. 10A, B, D). These results together suggest again that although cycling drugs in combination has the potential to limit rate of adaptation, the limitation appears to be influenced by more than simply the regimen.

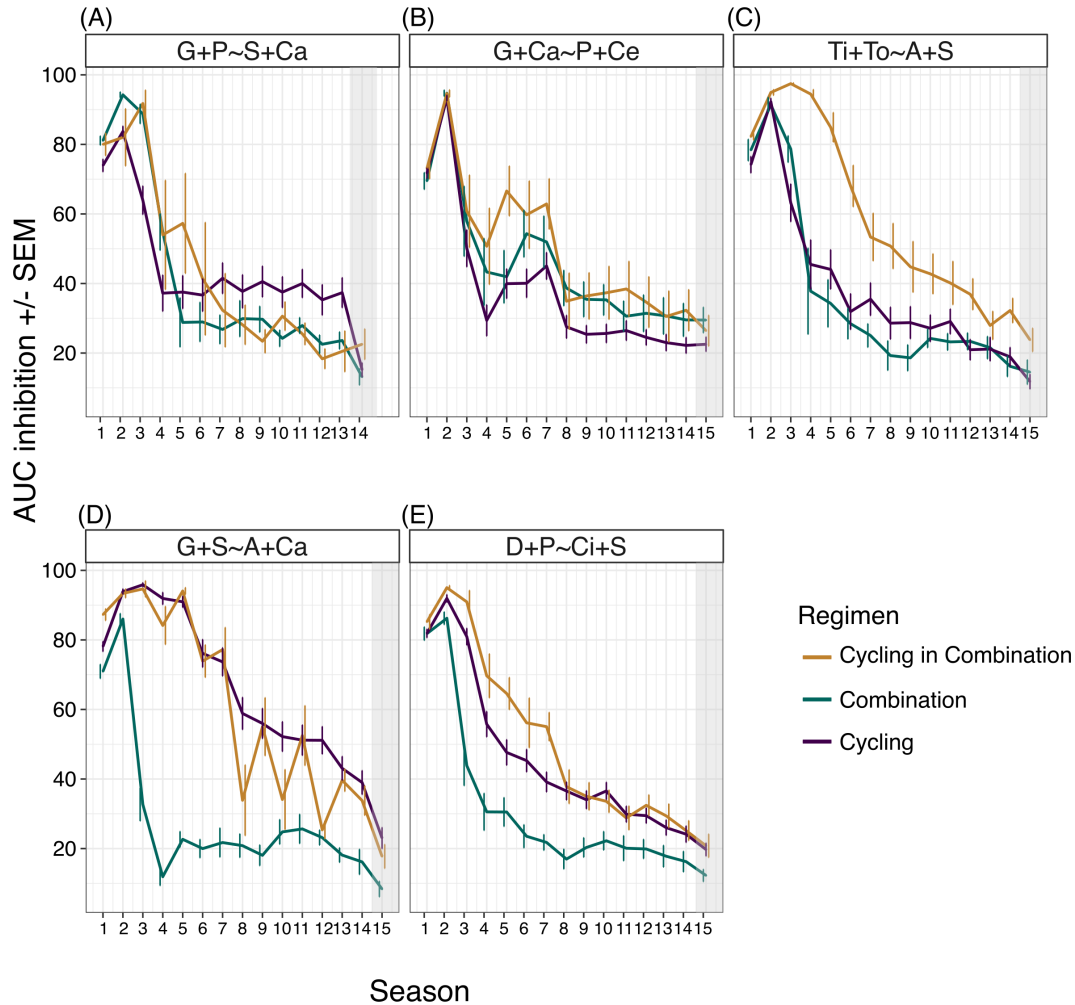


Figure 10 Evolutionary growth dynamics throughout evolution. Area under the curve inhibition (AUC_i, see Methods) was calculated per season per regimen first for each replicate, then for each treatment, and finally per regimen for each of the five evolution experiments (A-E). Error bars represent standard error of the mean (SEM) with the number of replicates per treatment group varying from 0 to 10, depending on the extinction rates, and the number of treatments within one regimen ranging from 2-8 (Fig. 5). Grey shaded area represents the no-drug season. Antibiotic abbreviations are shown in Table 2.

Cycling-in-combination regimen renders no visible increase in fitness costs

Thus far, evidence was provided that PA14 are able to adapt to almost every tested antibiotic therapy after ~5 days of evolution. Associated fitness costs were subsequently measured to determine if the ability to grow in these environments conferred an associated growth defect. To measure fitness costs, bacteria were grown for 24 hours in no-drug media directly following evolution. Bacterial lag time, growth rate, and maximum yield relative to the evolved, no-drug control ancestor were subsequently measured. As all measures showed similar trends, I will focus on relative maximum yield, though results of relative growth rate and relative lag time are displayed in the supplementary information (Fig. S5, Fig. S6).

All surviving populations generally showed a decreased maximum yield relative to the control evolved in no-drug media (Fig. 11). This finding demonstrates that bacterial populations able to sustain these high selective pressures had a corresponding cost to adaptation and were not able to grow to as high of a density in the absence of antibiotics. The extent of the decrease in maximum yield was also similar

across experiments, with the mean costs per regimen showing a mild defect in fitness relative to the evolved control. Notably, outlying populations were present that not only displayed no visible fitness cost, but also showed increased growing capacity in comparison to the evolved ancestor (Fig. 11). These outliers were also present in every evolution experiment, indicating that irrespective of the component drugs, bacteria could not only survive in these toxic environments, but did so without a measured cost in growth dynamics after 14 seasons of evolution. Although these populations were not the norm, they are important to identify as extremely fit populations could profoundly influence the experimental readouts and population dynamics.

Interestingly, when comparing fitness costs among regimens, there appeared to be no visible differences in every evolution experiment excepting one (Fig. 11C). In this single case, cycling drug combinations showed significantly more of a fitness cost than the two alternative regimens. However, the remaining 4 evolution experiments indicated no significant differences in relative maximum yield between the three regimens. Although cycling-in-combination adds a more rugged adaptive landscape, as generally seen in the extinction and growth inhibition data, no increase in resistance cost to this regimen was universally observed throughout the evolution experiments. This result implies that the costs to adaptation for this novel therapy are similar to those of cycling single drugs and drug combinations.

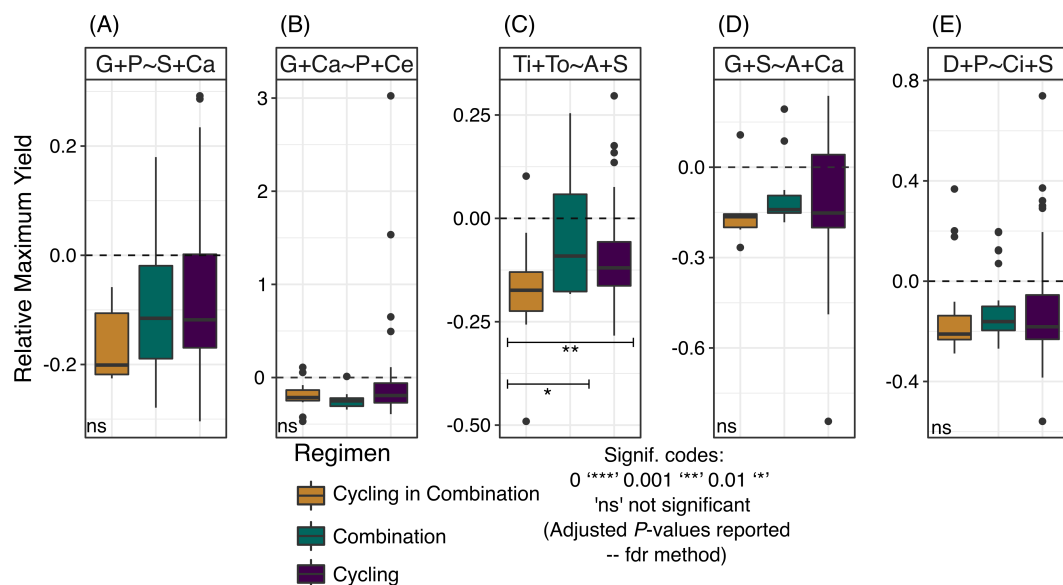


Figure 11 Relative maximum yield under antibiotic-free conditions (A-E). Maximum yield was calculated using the R package ‘grofit’²⁵⁹, and was made relative to the evolving control, evolving in no-drug medium. A linear mixed model was performed on each evolution experiment separately to test if the differences between the regimens were significant. Treatment was used as the fixed factor, relative growth rate as the response variable, and row in plate was a random factor. Differences between regimens were tested via a multiple comparisons post hoc test, and were corrected for multiple testing using fdr (see Materials and Methods). Antibiotic abbreviations are shown in Table 2.

Substantial variation within a single regimen highlights the influence of drug identity on evolutionary outcomes

Although summarizing the data by regimens is helpful to visualize general patterns, it is important to note substantial variations were present within regimens for each of the measured phenotypes. Treatments belonging to a single regimen often produced large variations in extinction rates, growth inhibitions, and fitness costs. To further explore the variation within a single regimen, each of the measured phenotypes

was subsequently grouped by treatment in attempts to investigate the magnitude of and underlying reason for said variation.

Treatments within one regimen displayed large variations in extinction rates (Fig. 12). These variations stem from very high, or in some cases, complete extinction present in one treatment nested within a regimen, while relatively little to no extinction present in the remaining treatment(s) in said regimen. The effect of antibiotic order had a striking impact in my extinction data, as large differences in extinction numbers were often observed in treatments containing the same antibiotics, applied in reverse order. For example, when cycling the drug combinations G+S and A+Ca, if the experiment started with G+S, the extinction percentage was ~37%, while starting with the A+Ca combination elicited 100% extinction (Fig. 12D). The same was seen for cycling between antibiotics G and Ca, in which starting with carbenicillin elicited 100% extinction, whereas the first season of evolution in gentamicin produced only 25% extinction (Fig. 12D). The order-effect observed in both cycling and cycling-in-combination regimens indicates that the drug choice, but also the schedule of deployment of chosen drugs are relevant in dictating extinction percentages in PA. Importantly, the order-effect was not present in every fluctuating environment tested, but seemed to be prevalent only in select fluctuating treatments.

To test if the high variation in extinction was due, in part, to drug interactions, previously attained FCR and PIS scores for the ancestor PA14 strain were obtained^{198,199}. As outlined above, drug interactions (neither FCR nor PIS) during combination therapy did not have a significant effect on extinction rate in my data. However, the presence of collateral sensitivity in drugs that are cycled has also been shown to have a large impact on treatment efficacy²⁴⁰. I therefore looked at associations between drug interactions and extinction rate specifically for the cycling regimen. All treatments that cycled single drugs for which collateral profiles were known were included (n=21). A GLM identified a significant association between both drug interactions (FCR and PIS) and extinction rate, suggesting that not only do evolutionary interactions affect extinction rate during cycling therapy, but physiological interactions also play a role in determining extinction rates (Fig. S4A, C, Table S13).

Variation between treatments belonging to a single regimen was also prevalent for growth inhibition measurements, though the variation appeared to be substantially less than the variance observed in extinction percentages (Fig. 12). While individual treatments within a regimen occasionally displayed fluctuations in growth inhibition depending on the drug treatment to which they were exposed, the extent of the observed differences in growth inhibition appeared to be lesser than observed in the extinction rates. Intriguingly, the order in which antibiotics were deployed appeared to have less of an influence on growth inhibition in the data, as large differences in growth inhibition of treatments containing the same drugs but deployed in reverse orders were never seen.

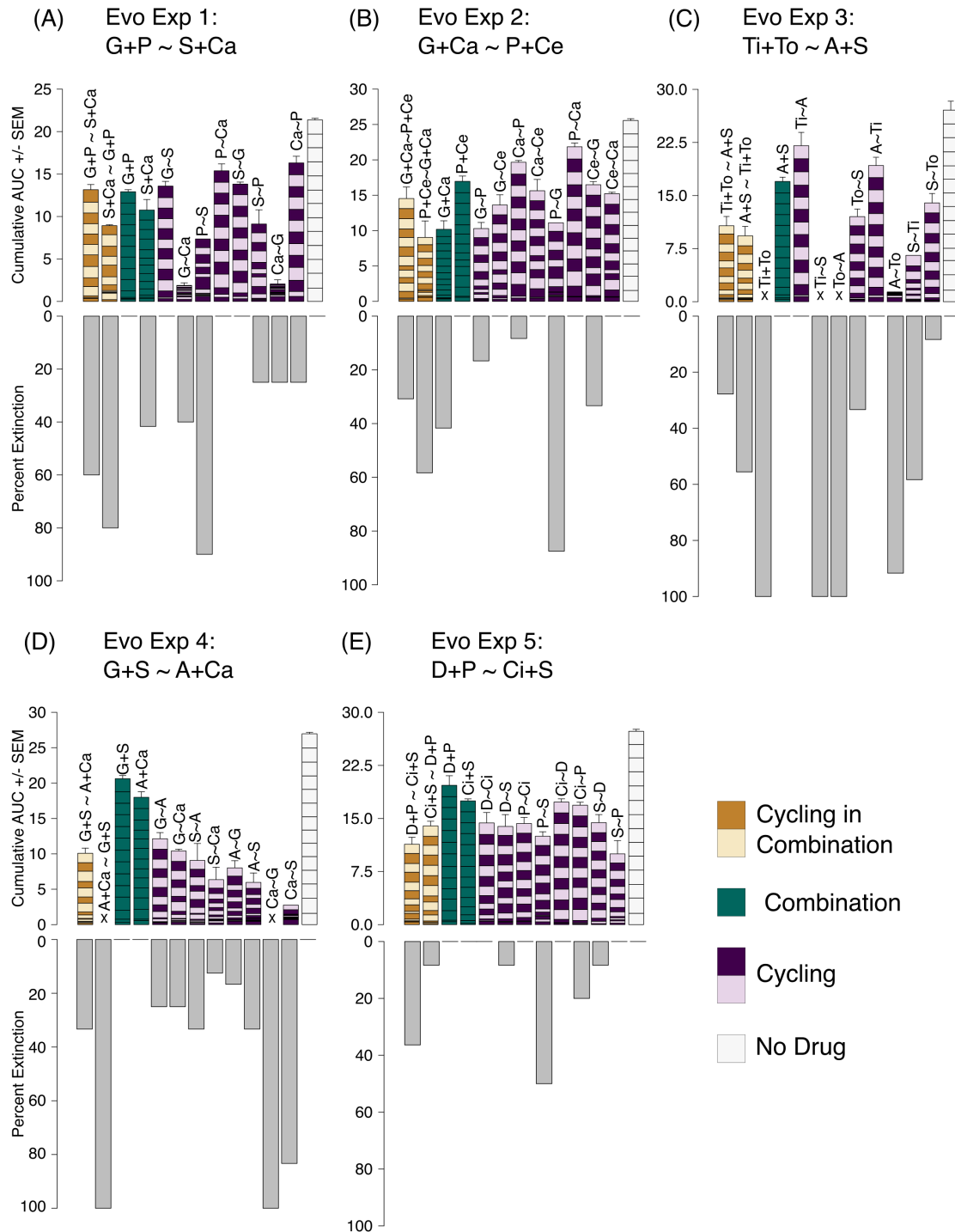


Figure 12 Cumulative AUC and extinction percentages for the individual treatments within the regimens of the five evolution experiments (A-E). Cumulative AUC was calculated for each replicate, and then each treatment type. The replicate number depended on the number of extinctions observed per treatment type, as extinct lineages were excluded from AUC calculations. Each stacked box represents the mean cumulative AUC of all replicates in a single treatment for one season. Error bars represent the SEM. The lower axes show the extinction rates per treatment (n=8-10). Antibiotic abbreviations are shown in Table 2.

To test if physiological or evolutionary interactions of the component antibiotics also influenced growth inhibition, mixed-effects linear models were performed separately for cycling and combination regimens. Although drug interaction did not seem to have a significant effect on extinction rate for treatments undergoing combination therapy, a significant association was found between both drug

interactions (FCR, PIS) and cumulative AUC measurements (Fig. S3, Table S13). This result shows that combinations interacting synergistically, but also to which the two component drugs display collateral sensitivity to one another achieved increased growth inhibition throughout evolution. Similarly, a significant association was also found between drug interaction (both FCR and PIS) and cumulative AUC for populations undergoing cycles of single drugs (Fig. S4, Table S13). This results again highlights that antibiotic pairs exhibiting both collateral sensitivity and synergism inhibit bacterial growth to a higher extent in both cycling and combination contexts.

Large variations in fitness costs also appeared in treatments belonging to one regimen (Fig. 13). As an example, the mean relative maximum yield of treatment S~P in experiment one is approximately -0.2, while treatment P~Ca displays a value of over 0.1 (Fig. 13A). Interestingly, treatments undergoing drug combinations cyclically tended to show similar fitness costs, each treatment showing a uniform decrease in relative maximum yield. However, the remaining regimens displayed fitness costs across the spectrum, with mean costs per treatment belonging to a single regimen ranging from strong decrease in fitness to increases in growth relative to the evolved ancestor. To test if these variations across treatments were due to physiological or evolutionary interactions, a Spearman's rank correlation was performed. No significant associations were detected between relative maximum yield and the component drugs' interactions (FCR and PIS) in neither cycling nor in combination regimens. The lack of a significant association signifies that these interactions had likely no measurable effect on the evolved population's fitness costs.

Importantly, variations did not only appear across treatments belonging to one regimen, but large variations were also prominent within populations undergoing the same treatment. For example, populations that underwent antibiotic treatment P~Ca in experiment one showed a mean relative maximum yield of ~0.1, but individual populations exposed to this antibiotic therapy range in this fitness proxy from ~-0.1-0.3 (Fig. 13A). This observed variation also appeared to be regimen-dependent, as there were relatively small variations within populations exposed to cycling-in-combinations, but large variations in populations undergoing both combination and cycling single drugs. This finding implies that treatments exposed to the cycling-in-combination regimen that had a higher selective pressure and more constrained adaptive landscape corresponded to paths to adaptation being limited and costly. While treatments undergoing drug combinations or cycling monotherapies could potentially take many paths towards resistance and thus display many costs including acquiring the ability to grow in these drug environments without a cost in measured growth rate.

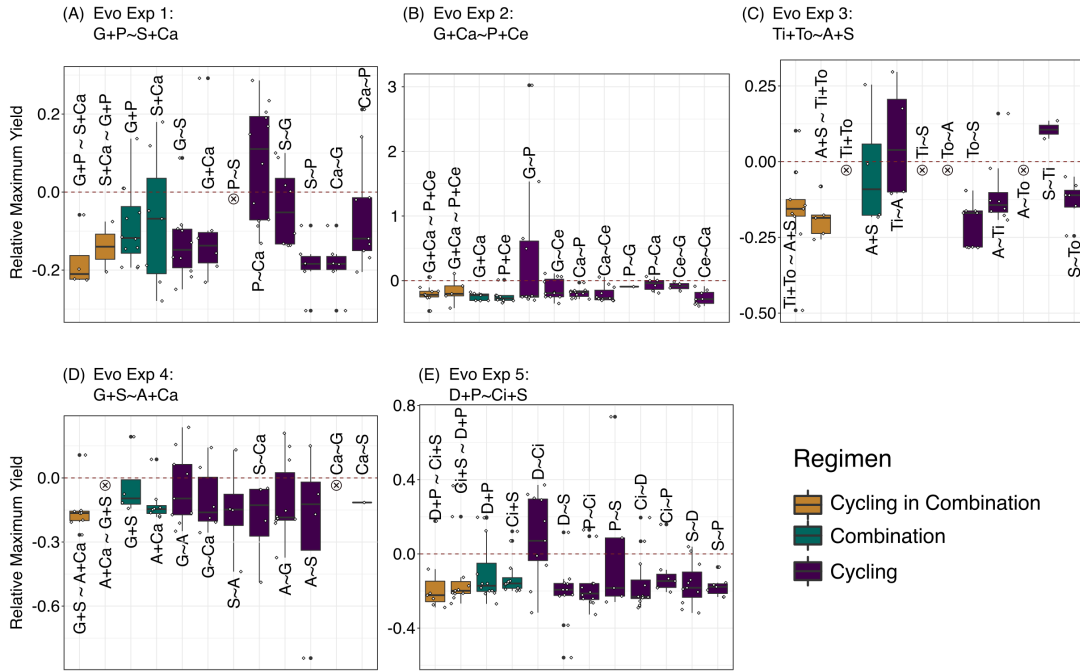


Figure 13 Relative maximum yield per treatment under antibiotic-free conditions in the five evolution experiments (A-E). Maximum yield was calculated using the R package ‘grofit’²⁵⁹, and was made relative to the evolving control, evolving in no-drug medium. Each white point represents the relative maximum yield of a single population (single bacterial well), and treatments that had no surviving lineages are marked with a cross. The number of replicates differs per treatment, as treatments showed different rates of extinction. Antibiotic abbreviations are shown in Table 2.

Heterogeneity within a single population suggests alternative evolutionary paths

As divergent fitness costs were observed between replicates, presence of increased variance among replicates in growth inhibition was subsequently tested. Evolutionary growth dynamics of single replicate populations and their relation to the treatment means were subsequently investigated. This allowed the identification of parallel or divergent evolution patterns observed in response to differential selective pressures.

Evidence for both parallel and divergent evolution was found in every evolution experiment (Fig. 14). While select antibiotic treatments created environments in which bacteria seemingly unanimously and quickly adapted, others caused individual populations to adapt at different rates. For example, populations exposed to the drugs G+P generally showed succinct growth dynamics, in that the populations were strongly inhibited in seasons 2-3, and the inhibition sharply declined from season 4-5 onwards (Fig. 14A). Each replicate in the treatment appeared to respond in this way, suggesting parallel evolution. Contrastingly, replicates subjected to the antibiotics A+S~Ti+To yielded high growth inhibition values until season 5-6, followed by select populations divergently adapting to the environment in season 7, 9, and 11 (Fig. 14C). Additionally, many populations seemed to show very low AUC_i throughout evolution, however, were able to grow proficiently in no-drug media. For instance, single wells in the treatments Ti+To ~ A+S (Fig 14C), S~P and D~Ci (Fig. 14E), P+Ce ~ G+Ca (Fig. 14B), A~S (Fig. 14D), and multiple wells in the treatments G~Ca, Ca~G (Fig. 14A), are inhibited approximately 100% by the antibiotic treatment, which is released during the treatment of no-drug media. These populations may be dormant for the entirety of evolution, and

only increase metabolism and growth when the adverse environments have been removed, suggesting tolerant or persistent growth in some populations. These results imply differential selective pressures brought about by the different regimens and treatments, cause the bacteria to either succinctly adapt, or struggle to find different paths to adaptation.

Regimen appeared to affect the dissimilarity seen between replicates exposed to a drug treatment, as PA14 exposed to drug combinations generally showed low variability with the exception of two treatments (S+Ca, G+Ca, Fig. 14A, B, respectively). Fluctuating environments predominantly led to more variation, with many treatments in each experiment showing strong diversity in the rate at which the populations adapt to the drug environments (Fig. 14). These fluctuating environments also produced what I term 'zigzag dynamics'. For example, populations exposed to D~S grew poorly when exposed to S, but growth would spike as soon as D was applied, and subsequently drop when again challenged with S (Fig. 14E, Fig. 15A). Zigzag dynamics were seen only in fluctuating environments, and were present in every experiment in at least one treatment. I thus proposed the presence of zigzags to be the result of interacting antibiotic collateral profiles. I hypothesized these patterns could arise from one of two circumstances. Firstly, non-reciprocal collateral profiles could generate zigzag dynamics. Taking the pair of drugs G and P as an example, the switch from G to P would theoretically lead to a decrease in AUC_i (decrease in growth inhibition) if the zigzags were due to collateral interactions, as resistance to gentamicin in most cases leads to collateral resistance in piperacillin/tazobactam in PA14¹⁹⁹. Contrastingly, switching from P to G would show an increase in AUC_i (increase in growth inhibition), as resistance to piperacillin/tazobactam generally leads to collateral sensitivity to gentamicin in PA14¹⁹⁹. Secondly, reciprocal collateral sensitivity could additionally cause zigzag dynamics if the population evolved resistance to one of two drugs in the fluctuating environment. A low AUC_i and thus proficient growth would be observed in the environment to which resistance had already evolved, while slow or dormant growth would be seen in the subsequent environment to which resistance had not yet evolved.

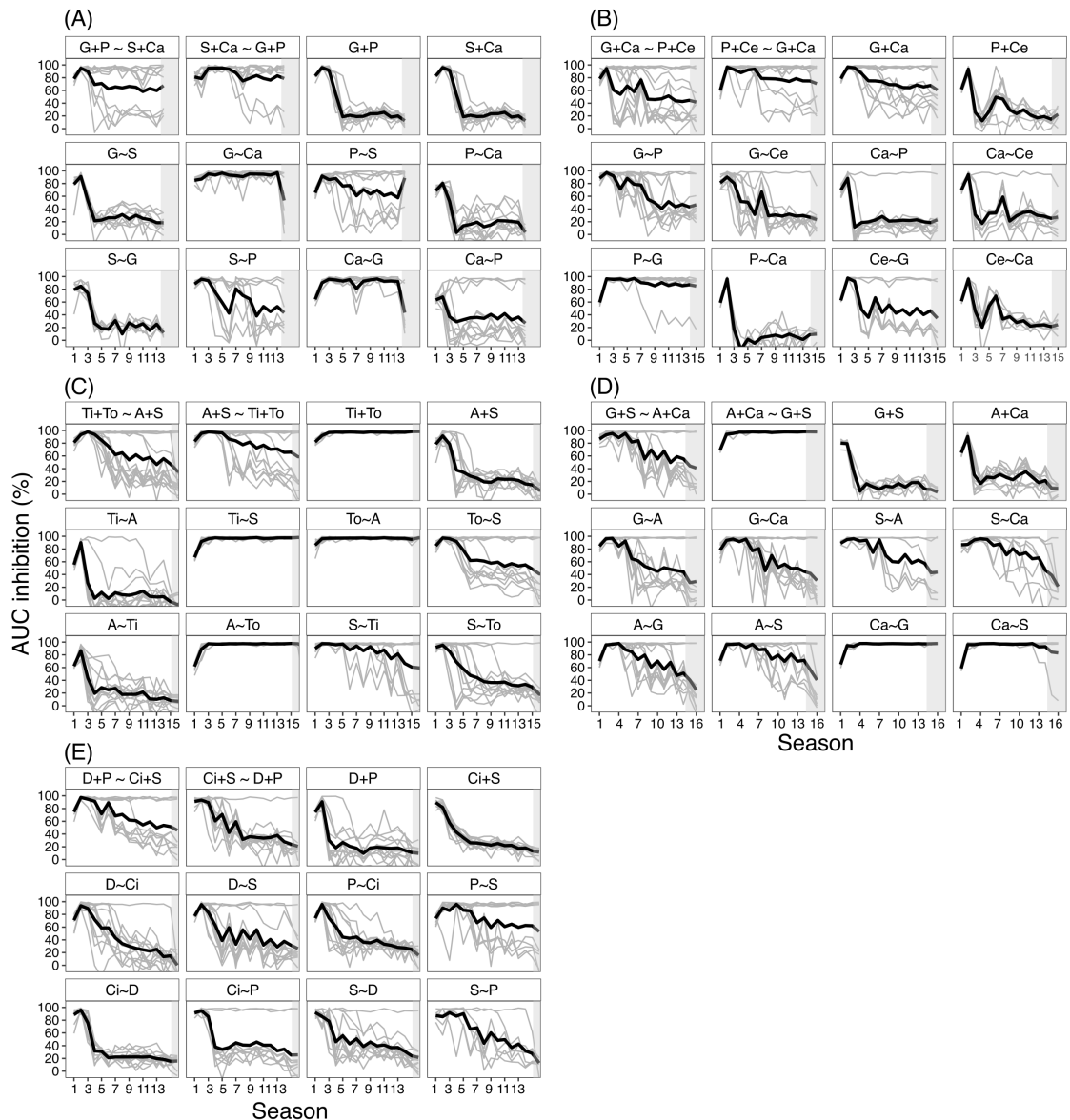


Figure 14 AUCi dynamics of each bacterial replicate population. Area under the curve (AUC) is expressed as a percent inhibition relative to the no-drug, evolving ancestor (see Materials and Methods). Grey lines represent replicate populations, and dark lines represent the mean of all replicate lines ($n=8-10$). Grey shaded boxes represent no-drug seasons. Antibiotic abbreviations are seen in Table 2.

To quantify the zigzag dynamics and to test the influence of collateral profiles on these patterns, the mean change in AUCi (ΔAUC_i) was calculated for every individual drug switch from seasons 5-11 (Fig. 15A). ΔAUC_i provides information both about the direction of the zigzag (negative values correspond to a decrease in inhibition during the switch from antibiotic 1 to antibiotic 2), and the extent of the zigzag (how large the difference in inhibition is during season 1 exposed to antibiotic 1 and season 2 with antibiotic 2) (Fig. 15A). Seasons 5-11 were chosen as zigzag dynamics were most prominent during these time points. Additionally, the majority of the drug therapies to which the populations quickly adapted already showed a large decrease in AUCi by season 5, suggesting that the majority of populations had adapted to the environment, leaving the remaining changes in AUCi likely due to differences in growth dynamics (Fig. 14). If zigzag dynamics were caused by non-reciprocal collateral profiles, I expected the ΔAUC_i values to be positive (corresponding to an increase in

growth inhibition) for the switches leading to collateral sensitivity, and negative for switches leading to collateral resistance. No evidence was seen that switches supposedly leading to collateral sensitivity showed exclusively positive ΔAUC_i values (Fig. 15B). Contrastingly, in most but not all cases, the ΔAUC_i values were positive for the switches corresponding to collateral resistance. The ΔAUC_i values displayed by pairs exhibiting reciprocal collateral resistance and reciprocal sensitivity were comparable to those of pairs with non-reciprocal collateral profiles, further suggesting that reciprocal collateral profiles were not the cause of these dynamics

If zigzag patterns were due to reciprocal collateral resistance, one would expect relatively small or no zigzag patterns in pairs displaying reciprocal collateral resistance, and large ΔAUC_i values in one of the two switches in drug pairs displaying collateral sensitivity. This was again not the case, as comparable ΔAUC_i values were seen in antibiotic pairs displaying reciprocal collateral resistance, collateral sensitivity, and non-reciprocal profiles (Fig. 15B). I thus concluded that the observed zigzag patterns were not due to collateral interactions. As divergent evolution was predominant between replicates exposed to the same drugs, another possible explanation for these patterns could be an additional layer of heterogeneity, namely the presence of subpopulations of bacteria within a single population. Select cells in a population could adapt to one environment, while only tolerating another, and lie in wait until the preferable environment is present. I subsequently continued to characterize select evolved material to test for evolved drug resistance and subpopulations.

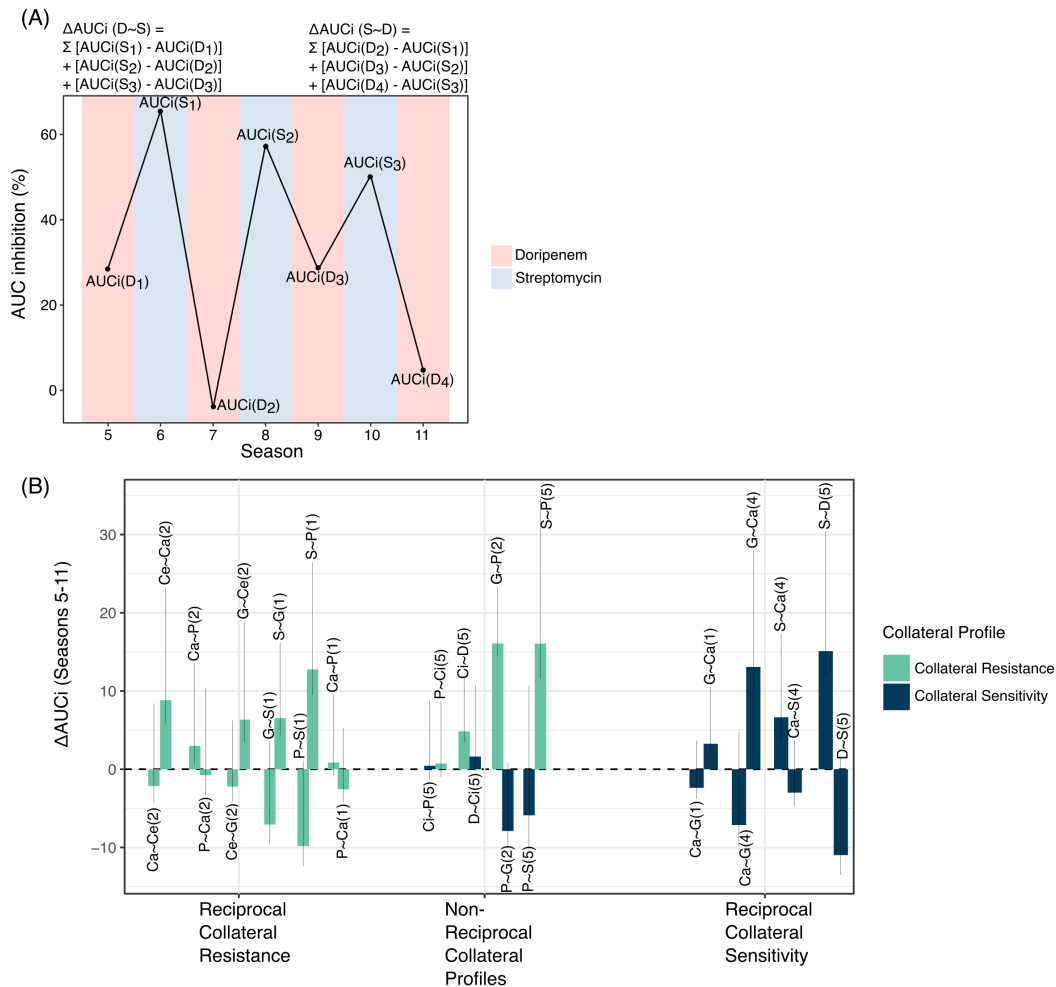


Figure 15 Change in AUC_i for seasons 5-11 for each antibiotic switch. (A) ΔAUC_i was calculated for each antibiotic switch. As an example, one population undergoing the antibiotic pair Doripenem and Streptomycin was used. When calculating ΔAUC_i for the switch from D→S, the differences in AUC_i were taken during the seasons containing S, from the previous season in D. This corresponds to the switch the bacteria see when they're coming from a D environment to a S environment, and how they react to this switch. The sum of all of the switches (from seasons 5-11) were calculated, and then averaged per switch and experiment (n=8-10). (B) Mean ΔAUC_i values are plotted for each switch and for each experiment (experiment number seen in parentheses) (n=8-10). If the antibiotic combination was tested more than once, the bars are shown separately, as each experiment showed differing dynamics, and error bars represent standard error of the mean. Antibiotic abbreviations are shown in Table 2.

Population diversity increases in response to cycling-in-combination therapy

Select populations were chosen from each regimen in evolution experiment 5 for subsequent characterization following evolution (Table 3). Experiment 5 was chosen, as this experiment contained many treatments showing zigzag dynamics, but also treatments displaying more uniform evolution as a comparison (Fig. 14). A total of 8 treatments were chosen, and 3 populations (3 replicate wells) from each treatment were included based on their similarity to the population mean (Table 3, Fig. S8). I aimed to chose differing replicate populations, one displaying similar growth characteristics to the AUC_i treatment mean, one above, and one below the AUC_i treatment mean (Fig. S8). Frozen material from seasons 7 and 14 for each of the 3 populations was thawed and streaked on agar plates. 20 colonies were subsequently

randomly chosen from each population, and challenged with a gradient of antibiotic concentrations to investigate phenotypic resistance of isolates in a single population (Fig. 7).

Population heterogeneity was analyzed by first performing a hierarchical clustering analysis measuring Euclidian distance between the changes in resistance for two tested drugs and for each colony extracted from two time points of evolution (see Methods). The presence of bacterial subpopulations was identified in every drug regimen, suggesting that bacteria contained in a single well can evolve differentially to a selective pressure (Fig. 16A). The number of subpopulations per population and the number of tested populations containing phenotypically distinct clones appeared to increase in fluctuating environments, as the majority of wells belonging to cycling and cycling-in-combination therapies displayed minimally 2 subpopulations. Populations exposed to combination therapies showed a maximum of two subpopulations, and many populations contained only a single phenotypic variant, suggesting that stochastic environmental changes favor an increase in population heterogeneity, while static environments encourage more constrained paths to resistance. To test this theory, a Shannon's Diversity Index was calculated from the clustering dataset. Cycling drugs in combination significantly increased population diversity when compared to combination therapy ($Z = 2.717$, P -value = 0.0197) and trended towards increased diversity compared to cycling single drugs ($Z = 1.99$, P -value = 0.07, Fig. 16B). However, cycling single drugs did not appear to significantly increase population diversity when compared to combinations ($Z = 1.15$, P -value = 0.25, Fig. 16B). This finding implies that applying drug combinations cyclically favors population heterogeneity when compared to the alternative regimens, and thus seemingly fostering divergent evolution within a single population.

The detected subpopulation frequency also differed depending on the time extracted during evolution, with populations showing both increases and decreases in subpopulation numbers from season 7 to season 14 (Fig. 16A). Interestingly, five cases showed a decrease in population diversity from time point 7 to 14, while six showed an increase in population divergence during the later seasons. Intriguingly, if cycling drug combinations displayed differing subpopulation numbers throughout evolution, an increase of time spent in evolution lead to a decrease in population variation. A single population challenged with a combination therapy showed an increase in population divergence, while cycling single drugs supported both the increase and decrease in population variance, with some populations exposed to the same drugs generating both increasing and decreasing diversity over time. These results suggest a convergent and divergent evolution can be prompted by the same regimen and treatment.

I additionally tested if population heterogeneity could explain the previously identified growth zigzag dynamics observed during evolution. When comparing presence and number of subpopulations within a single well, no observable patterns were detected to illustrate that zigzag dynamics were the result of subpopulations (Fig. S9). For example, the populations exposed to the drug combination Ci+S during evolution displayed comparably uniform growth dynamics (Fig. 14E, Fig. S8), however while 2 out of 3 tested populations showed no presence of subpopulations, population 1 showed phenotypic variations resulting in 2 subpopulations during seasons 7 and 14 (Fig. 16A). Contrastingly, populations undergoing the cycling treatment D~S had comparatively large zigzag dynamics present in every tested population (Fig. 14E, Fig. S8), and while population 1 contained up to 3 subpopulations during season 7, population 2 contained no subpopulations, and population 3 only generated subpopulations during season 14 (Fig. 16A). I thus statistically tested if the presence or

number of subpopulations correlated with extent of zigzag dynamics. The previously calculated ΔAUC_i was subsequently taken as a proxy for zigzag dynamics presence and extent and, using a Spearman's rank correlation, no evidence was found that number of subpopulations ($\rho = 0.133$, P -value = 0.52, Fig. S9A) nor presence or absence of subpopulations ($\rho = 0.078$, P -value = 0.71, Fig. S9B) correlated with zigzag dynamics. These results indicate that although prevalent presence of subpopulations was found in our data, there is not sufficient evidence to conclude that subpopulation presence or number caused the zigzags seen during evolutionary growth dynamics. However, it is important to note that the characterization of evolved material has limitations including: (1) the prevalence of the subpopulations needed to be high enough in order for their detection; (2) the populations needed to display a high amount of phenotypic differences in their responses to the antibiotics to be classified as a "subpopulation"; (3) only seasons 7 and 14 were tested, and if the populations were exposed to fluctuating environments, the antibiotic(s) given during season 7 were different to those in season 14. To give an example, for a population being challenged with the fluctuating environment D~S, said population had been extracted after being exposed to D during season 7, and after being exposed to S in season 14. The population could theoretically select for resistant clones to D in season 7, and S in season 14, and depending on the drug seen in the isolated season, could bias the subpopulation results. As exposed selective pressure would select for clones more resistance to that drug and select against remaining subpopulations, further analyses involving characterization of material from subsequent seasons would be required. These experiments were unfortunately not in the scope of this thesis, and as this was not the main question, we continued to analyze resistance profiles of the already-characterized evolved material.

Table 3 Selected treatments chosen to undergo further characterization

Regimen	Antibiotic Treatment
Cycling in combination	D+P ~ Ci+S
Cycling in combination	Ci+S ~ D+P
Combination	D+P
Combination	Ci+S
Cycling	D ~ Ci
Cycling	Ci ~ D
Cycling	D ~ S
Cycling	S ~ D

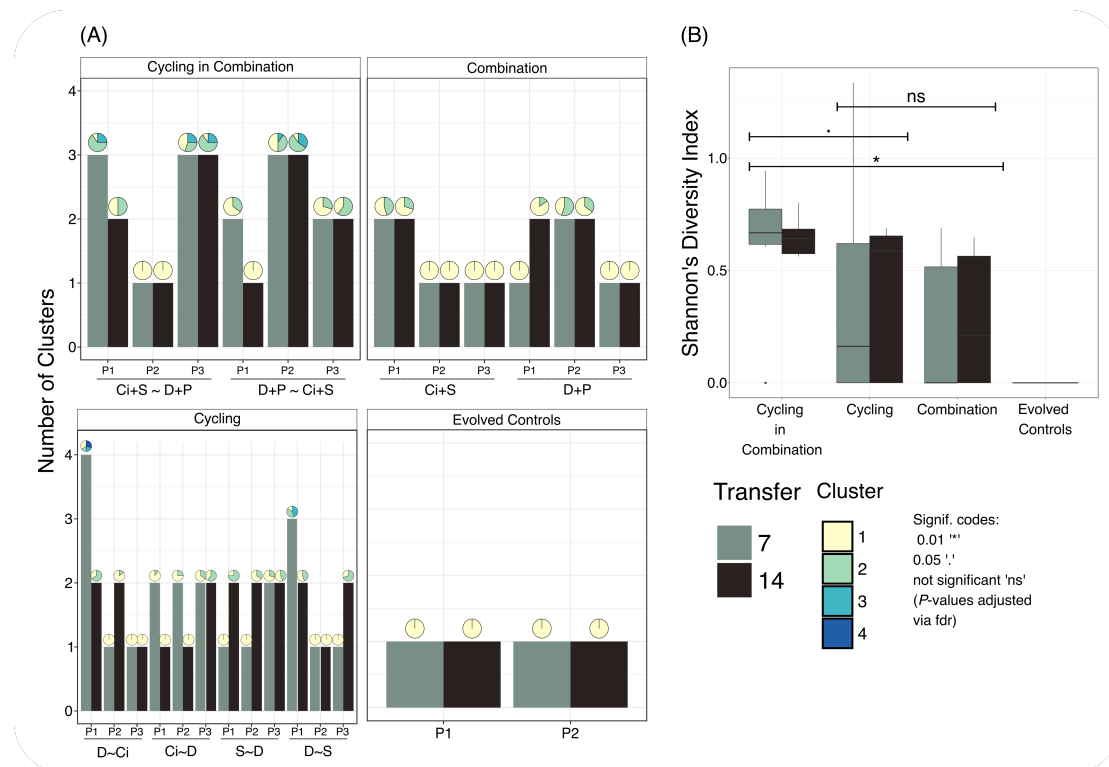


Figure 16 Maximum cluster number per well for evolved populations. (A) Select evolved material was characterized by challenging 20 colonies from a single population (denoted as P1, P2, P3) to increasing concentrations of antibiotics (see Methods). Hierarchical clustering analyses were then performed to determine population heterogeneity. Clustering was performed per antibiotic, and the maximum number of clusters identified for each population is shown on the y-axis. Pie charts above the bar plots show the proportion of tested colonies belonging to each cluster. (B) Shannon's diversity index was calculated per regimen with the clustering data to test for differences in population heterogeneity between regimens. Population heterogeneity differences between regimens were assessed via a linear model, and probability adjustment was based on the false discovery rate (fdr) to account for multiple testing.

Cycling drugs in combination does not generate an increase in multidrug resistance

Changes in resistances relative to the PA14 ancestor were quantified to investigate resistance profiles of individual clones and to assess the prevalence of multidrug resistance. Changes in resistance were calculated for each of the 20 chosen colonies and for each of the antibiotics seen during evolution. PA14 ancestor was used as the reference strain (see Methods). Resistance patterns varied substantially within regimens, treatments, and individual populations. The presence of increased resistance was prevalent for every regimen and at each time point, but importantly, increased sensitivity compared to the ancestor was also regularly observed (Fig. 17). Divergent evolution was again common both within and between populations exposed to the same antibiotic therapy and in both tested seasons. For example, the colonies exposed to D+P~Ci+S during evolution showed large variations in their resistance to both P and to D in season 7, with some clones within a single population displaying high resistances, and others high sensitivities (Fig. 17A). In contrast, colonies exposed to D~S during evolution showed more uniform resistance responses within a single population during season 7, however population 1 showed general sensitivity to both of the tested drugs, while population two boasted high resistance values to both antibiotics, and population 3 landed somewhere in the middle, with light increases in

resistance seen for the drugs. These results again highlight the presence of different paths to survival observed in the evolution experiments.

Focusing on how a single colony evolves resistance to survive in different drug therapies, reveals several emerging trends. Firstly, in fluctuating environments, resistance generally evolves to a part of the treatment, meaning one drug for cycling monotherapies and one or two of the four drugs for cycling drug combinations. This partial resistance implies that the cells are proficient in growing in one environment, while somehow tolerating the remaining environment, waiting for the preferred drug to again emerge. Secondly, there are several cases in which high resistance to both administered drugs is present in cycling therapies (D~S, S~D, D~Ci) and in one combination therapy (Ci+S). While multidrug resistance is not generally prevalent in our dataset, resistance to multiple drugs frequently occurs in the D+P combination therapy, and in select clones during later timepoints for both fluctuating environments. Thirdly, select populations withstand the administered drugs without acquiring any additional resistances, even throughout late seasons of evolution. A particularly interesting case is seen in the combination environment D+P, which largely remains tolerant to both of the challenging drugs throughout the entire evolution experiment. Although several clones were present that displayed no resistance to the challenged drugs in season 7 for both fluctuating environments, only a few populations in the Ci+S~D+P treatment, select clones in the S~D treatment, and, surprisingly, the majority of clones tested in the D+P treatment evolved no resistance to the tested drugs following season 14.

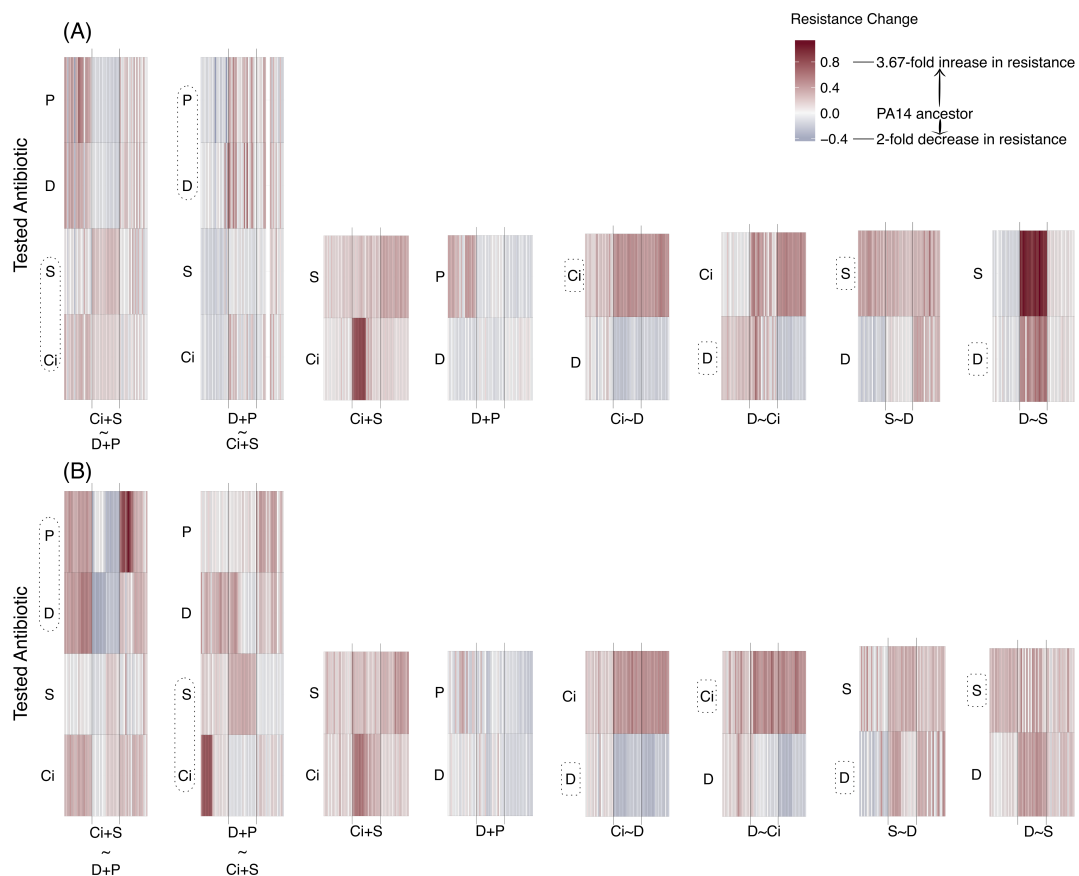


Figure 17 Evolved material change in resistance relative to the ancestor during seasons 7 and 14 of evolution. Frozen material extracted during season 7 and 14 was thawed and streaked on agar plates. 20 colonies were randomly chosen from the specified populations belonging to each antibiotic treatment. The clones were then challenged with increasing concentrations of antibiotics previously administered during evolution. The change in resistance was then

calculated in reference to the PA14 ancestor strain (see Methods). Each bar represents the change in resistance for a single colony to the tested antibiotics, shown on the y-axis. For the fluctuating environments, the circled antibiotic(s) are the drugs which the population had been exposed to immediately prior to freezing. Panel (A) represents measurements taken from season 7, and panel (B) shows results from material isolated from transfer 14.

As multidrug resistance is a particularly undesirable phenotype in terms of resistance evolution, but was nevertheless observed in several treatment types, the prevalence of multidrug resistance observed in each regimen was subsequently tested. A multidrug resistance index was calculated for each characterized evolved clone (see Methods). In short, the integral of the changes in resistances relative to the PA14 ancestor for each drug at each concentration were summed and then divided by the total number of tested drugs. Interestingly, cycling drugs in combination showed no increases in multidrug resistance when compared to drug combinations, suggesting that although this regimen adds two new drugs to the therapy, the presence of the additional drugs does not select for more resistance (P -value > 0.997 , Fig 18B). Cycling single drugs, however, significantly increased prevalence of multidrug resistance when compared to both combination (P -value < 0.001) and cycling-in-combination (P -value < 0.001) therapies (Fig. 18A). Although this result appears to be largely influenced by a few outliers which show large MDR indices, it is nevertheless significant because MDR rates have been previously shown to decrease during cycling therapy²³⁸.

As the evolved material was isolated during the middle and end of the evolution experiments, the influence of time spent in evolution on the resistance profile of individual isolates was subsequently investigated. When comparing material isolated after season 7 to that of season 14, a general increase in resistance prevalence and extent is seen during the later season of evolution for individual colonies (Fig. 17). It was first speculated that the drug used in the final cycle before isolation would influence the resistance patterns seen in isolated material. However, this does not appear to be common in the dataset. Specifically, the cycling therapy Ci~D shows high resistance levels to Ci following season 7, where the challenged antibiotic was Ci (Fig. 17A). However, these resistance trends remain following season 14, after being exposed to D for 12 hours (Fig. 17B). A similar result is seen for the antibiotic treatment S~D and for both treatments in the cycling-in-combination regimen (Fig. 17). Although there was notably more resistance in some of the treatments following season 14, we could not distinguish whether that increase in resistance stemmed from the increased time in evolution or the difference final antibiotic exposure.

To further investigate the influence of season of isolation on resistance profile, a mixed linear model was fitted to changes in resistance for single and multiple antibiotics and tested for significance. For single drugs, although there was a trend to increasing resistances present following season 14 in every antibiotic and regimen, only Ci showed significantly different changes in resistance from season 7 to season 14 (Fig. 18B, Table S10). Although multidrug resistances generally also increased from season 7 to season 14, this increase was also not significant for MDR indices (P -value < 0.626). These results interestingly indicate that both single and multidrug resistances do not significantly increase their drug resistance with increased time spent in evolution.

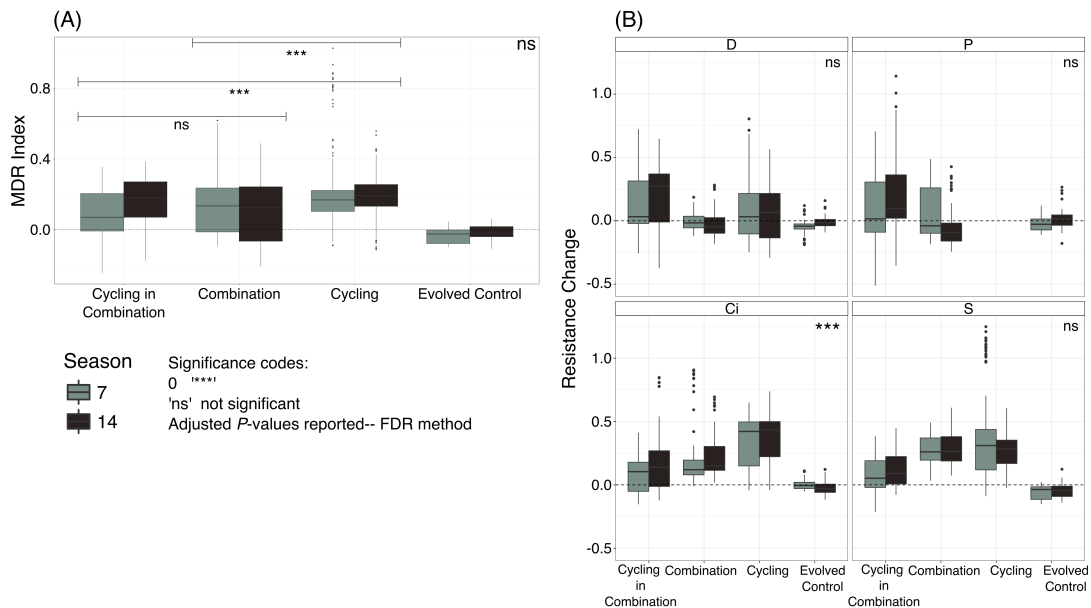


Figure 18 Change in single and multidrug resistance throughout evolution. (A) Multidrug resistance was calculated for each isolate clone relative to the PA14 ancestor (see Methods). A mixed linear model was subsequently utilized to assess differences in multidrug resistance prevalence between regimens, and *P*-values were corrected for multiple comparisons via *fdr*. An additional mixed linear model was performed to assess if MDR indices differed between isolated seasons, which show non-significance in the top right panel. *P*-values were corrected for multiple comparisons via *fdr*. (B) To further explore the influence of time spent in evolution on the resistance evolution of individual antibiotics, the change in resistance relative to the PA14 ancestor was calculated for each isolated clone and means of all colonies belonging to a single regimen are plotted in panel above. Subsequently, a mixed linear model was performed per antibiotic to determine if drug resistance differed between seasons 7 and 14 (see Methods). Reported *P*-values in the top right corner of each panel show presence or absence of a significant difference between resistance changes in season 7 and season 14, assessed by HSD following the mixed linear model, and were corrected for multiple comparisons via *fdr*.

Fitness costs of evolved populations show no correlations with resistance levels

To test the evolutionary costs of resistances, the fitness of each of the characterized colonies isolated from evolved material was measured. Frozen stocks of isolated clones were first allowed to preadapt to liquid media, then grown in no-drug media for 16 hours. Growth was monitored using continuous OD₆₀₀ measurements, and the PA14 ancestor simultaneously grown was used to calculate the relative maximum yield, lag time, and maximum yield of each isolated colony (see Methods).

Both decreased (increased time in lag phase, decreased growth rate, reduced maximum yield) as well as increased clonal fitness was detected in colonies belonging to each drug regimen (Fig. 19). Higher degrees of fitness change relative to the ancestor were more prevalent in the fitness proxies lag phase duration and growth rate, suggesting that bacterial maximum yield is affected to a lesser extent by the acquired resistances in our experiments (Fig. 19). Additionally, these high fitness costs and benefits also appeared primarily in the cycling-in-combination and the combination regimens, though select colonies in the cycling regimen also displayed high changes in fitness. These results imply that the resistances evolved to grow in cycling-in-combination and combination drug therapies generally carry a larger cost or benefit to the colonies.

Interestingly, the measured parameters used to estimate fitness also yielded strong differences in amount of colony variation and direction of the cost (increased or decreased fitness). For example, while most colonies in population 2 exposed to

Ci+S~D+P during evolution showed a large increase in the time spent in lag phase and a decrease in growth rate (Fig. 19A, B), the colonies displayed minimal changes in the maximum yield relative to the PA14 evolved control. The amount of clonal variation also depends on the measured fitness proxy. Population 1 exposed to D+P during evolution generated large variations between colonies for relative growth rate and maximum yield measurements (Fig. 19B, C), but colonies stayed in lag phase for similar time periods (Fig. 19A). These results indicate that the measured fitness proxies differ in their ability to estimate fitness costs present in bacterial populations for our characterized material. To further understand the relationship between the calculated fitness parameters, fitness proxies were correlated to one another. Relative length of lag phase yielded a mild negative correlation to both growth rate and maximum yield (Fig. 19D,F), and a slight positive correlation was observed between growth rate and maximum yield (Fig. 19E).

The data was again heterogeneous, with populations and clones within a population displaying deviations in relative lag time, growth rate, and maximum yield (Fig. 19). For example, colonies in population 2 exposed to D+P~Ci+S produced both large increases and decreases in duration of lag phase (Fig. 19A). Although select populations produce uniform fitness costs across colonies, discrepancies in colonies belonging to one population were universally seen throughout the dataset in all of the measured proxies for fitness. To further test if these large fitness costs were associated with specific resistances, each fitness parameter was correlated with change of resistance per antibiotic and multidrug resistance indices. Single resistances associated with each of the tested antibiotics were first analyzed against each fitness proxy. Although several weak correlations were present, the extent of the change in resistance did not significantly increase fitness costs for any of the measured drugs in any of the measured proxies for fitness (Fig. S10). MDR indices similarly failed to yield any prevalent correlations with the measured fitness traits (Fig. S11), though relative growth rate showed a mild negative correlation ($\rho = -0.22$, P -value < 0.001), suggesting a multidrug resistance could have a mild effect on bacterial growth rate during my experiments.

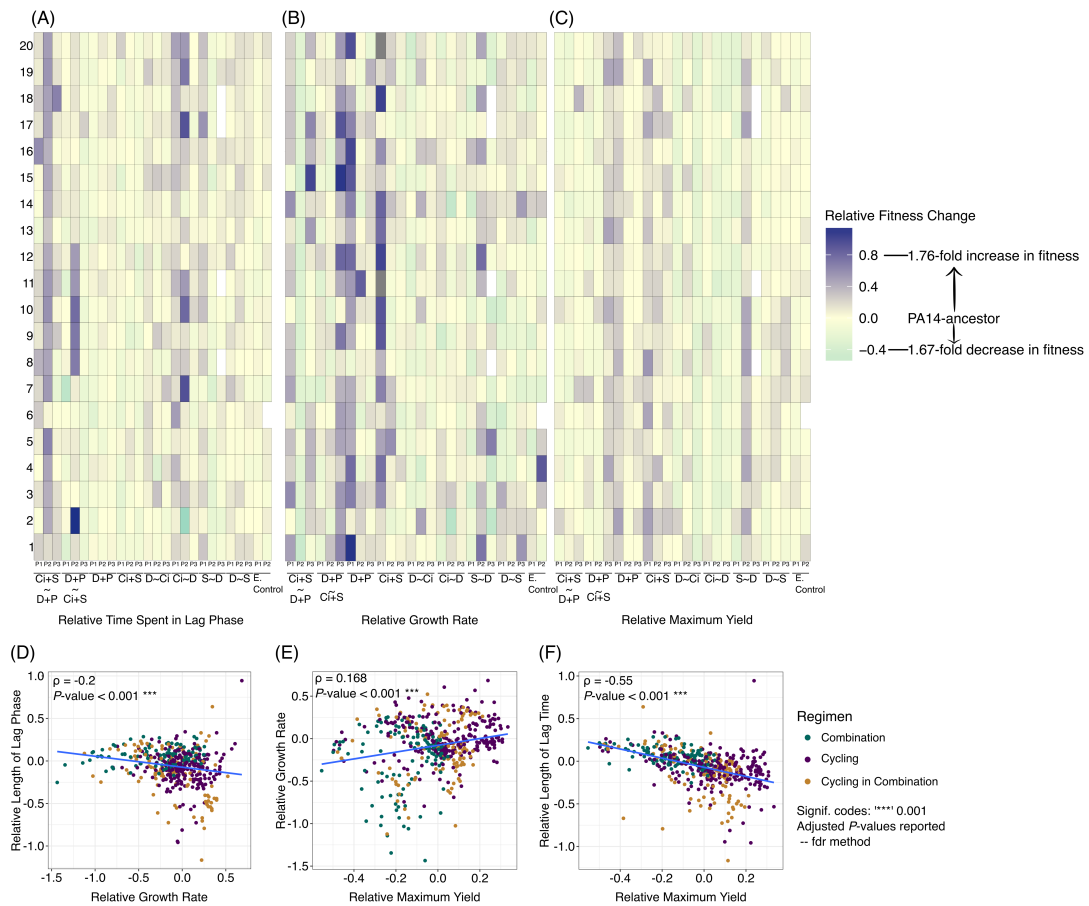


Figure 19 Relative fitness measurements for characterized evolved material. Previously isolated, frozen clones were thawed, subjected to a 3-hour pre-adaptation phase in no-drug media, then subsequently diluted into fresh M9 medium, and growth dynamics were measured for 16 hours, with continuous OD_{600} measurement. (A-C) Relative duration of lag phase, relative growth rate, and relative maximum yield were calculated using the package ‘grofit’ in R and were taken relative to the PA14 ancestor. (D-F) Spearman correlations were calculated to compare each of the fitness measurement proxies to one another. Each point represents one colony tested, the regression line is shown in blue, and the Spearman correlation coefficient as well as associate P -values are shown in the top left corner of the panels, and are adjusted by fdr for multiple comparisons

Discussion

The increasing prevalence of multi and extensively drug-resistant (MDR, XDR) pathogens threatens the efficacy of modern medicine, as formerly-effective antibiotics are, at times, failing to treat even simple bacterial infections⁹. *P. aeruginosa* is a significant contributor to these trends, with frequencies of MDR and XDR strains reaching from 15-30% in some areas²⁷²⁻²⁷⁴. As resistance rates increase and efficacy of single antibiotics decrease, the importance of researching alternative strategies to treat infections is paramount. During this thesis, I performed a series of *in vitro* evolution experiments with the model organism *P. aeruginosa*. Experiments compared evolutionary outcomes and dynamics between 3 multidrug antibiotic therapies including cycling drugs, drug combinations, and a novel therapy which has, to my knowledge, never before been tested, cycling drug combinations. I hypothesized cycling drugs in combination to further limit bacterial evolution based on 3 main criteria. Firstly, the heterogenous environment brought about by cycling drug combinations imposes a fluctuating, rugged adaptive landscape that further complicates evolution by interrupting selective sweeps. Secondly, fluctuation also allows for the addition of 4 drugs without the concatenate increase in drug concentration, which could otherwise be toxic for patients. Lastly, increasing number of component drugs likewise increases the number of physiological and evolutionary interactions able to be exploited, further propagating the therapeutic potential to decrease rate of adaptation.

It is important to note that these alternative therapies are not intended for simple infections easily treated by conventional antibiotic monotherapies. These treatments are rather directed towards chronically ill patients who require constant antibiotic exposure, thus specifically requiring treatments to select against multidrug resistance (for example, patients suffering from Cystic Fibrosis). However, I am also aware of the danger of drawing parallels between *in vivo* and *in vitro* experiments, which take place in a very controlled environment, without the presence of immune cells, competitors, and additional stressors. Therefore, the application of these therapies to patients would require many additional *in vivo* and *in vitro* experiments to assay bacterial responses in more complex environments. Nevertheless, the controlled setting of lab experiments provides valuable insight into determining how specific selective pressures drive different evolutionary paths to resistance. Understanding how different stressors select for specified paths to resistance is an informative building block for designing rational therapy, with the ultimate goal of deploying drug therapies that invert the selective advantage of multidrug resistant bacteria^{58,242}.

This study systematically compares the evolutionary outcomes of 3 alternative therapies by measuring distinct bacterial phenotypes from 5 independent evolution experiments during and following evolution. The use of multiple phenotypes to measure treatment efficacy further dissects the multifaceted consequences that each alternative therapies has on bacterial evolution. In the following, I will first discuss results from preliminary experiments, specifically the inability to standardize evolution experiments when using longer incubation times. I will then focus on the results of my evolution experiments, namely the 4 phenotypes chosen to assess antibiotic treatment efficacy (bacterial extinction, growth inhibition throughout evolution, fitness costs, and rate of multidrug resistance). Lastly, I will explore the result of experiments following my evolution analyses which highlight bacterial diversity within a single replicate population.

Effects of season length on bacterial growth

Prior to starting the evolution experiment, inhibition concentrations were standardized in order to effectively compare between antibiotic regimens during and following experiments. I found that the amount of drug needed to inhibit *P. aeruginosa* to 50% increased for every tested antibiotic when a season length of 24 hours was used (Fig. 8). An MIC increase with increased incubation times using broth microdilution method has also been observed in many lactic acid bacteria, for which an increase in incubation time from 24 to 48 hours resulted in a twofold increase in MIC²⁷⁵. This required increased drug concentration could be the result of 1 or a combination of 2 hypotheses. Firstly, decreased bacterial killing could be the result of antibiotic degradation effects. Antibiotic stability is known to be dependent on various conditions including temperature, pH, light exposure, and medium conditions²⁷⁶. A recent study reported 44.7% of cefotaxime, a third-generation cephalosporin similar to cefsulodin used in my experiments, was degraded after 24 hours of 37°C incubation in Mueller-Hinton broth²⁷⁷. Contrasting results report antibiotic efficacy remains stable over 6 weeks, however these experiments notably took place in saline²⁷⁸. Interestingly, gentamicin was the only drug to show 25% degradation during this study²⁷⁸, which is relevant for my data, as gentamicin was also the only tested antibiotic in my data set that elicited a significant increase in the IC₅₀ during 24-hours (Fig. 8B). As the half-life of all tested drugs was above 12 hours in these experiments, increased antibiotic degradation at 24 hours is a viable hypothesis to explain the increased drug concentration required to achieve 80% bacterial inhibition²⁷⁷.

A longer time spent in stationary phase could additionally explain the increased drug concentration required for bacterial inhibition. As PA14 generally transitions into stationary phase growth in M9 media in ~9 hours²⁰¹, the 24 hour season length led to a considerable increase the time spent in stationary phase. As all of the utilized antibiotics require fast growth in order to be effective, the transition into slow growth during stationary phase has been shown to increase bacterial tolerance to antibiotics²⁷⁹. In addition to slower growth, many downstream bacterial metabolic factors alter during stationary phase, which can further perpetuate bacterial tolerance to antibiotics²⁸⁰. For example, superoxide dismutase, an enzyme that catalyzes superoxide radicals in the bacterial cell, has been shown to confer multidrug resistance in *P. aeruginosa* during stationary phase by decreasing membrane permeability and drug penetrance²⁸¹. Although the time spent in lag phase in my experiments may seem negligible compared to environmental and clinical samples, this increase incubation time could account for the higher drug concentration needed to inhibit bacteria during our experiments.

In addition to higher antibiotic concentrations needed to elicit bacterial inhibition, an increase in the variation seen between replicates and between experimental runs additionally increased (Fig. 8 A, B). Carbenicillin generated high between-replicate variation, while the remaining antibiotics produced large differences in IC₅₀ values depending on the day in which the experiments took place (Fig. 8). The large amount of variation seen between replicates could be due to an increased phenotypic heterogeneity, as populations which spent longer time in stationary phase have been reported to increase the amount of population phenotypic heterogeneity²⁸². Although population heterogeneity during stationary phase has been widely studied using longer (days to weeks) batch cultures²⁸³, additional studies have found strong phenotypic differences in shorter-term (24-36 hours) stationary cultures of *E. coli*^{284,285}. For example, the rate at which single cells “reawaken” to tolerate antibiotics differed when stationary cultures of *E. coli* were transferred to fresh media²⁸⁵. This was shown to be due to an increased rate of persister cell formation when cells were kept in

stationary growth phase for 24-36 hours²⁸⁵. Persister populations have been shown to consist of several subpopulations in the context of tuberculosis, each with different cellular physiologies and corresponding antibiotic tolerances²⁸⁶. As *P. aeruginosa* also regularly forms persister cells, particularly in cystic fibrosis patients, where the bacteria likely spend the majority of their life in stationary phase²⁶⁹, the increased formation of persisters and thus increased population heterogeneity formed during increased time spent in stationary phase is a probable explanation for the increased variation seen in my data during incubation times of 24 hours.

During preliminary experiments, I detected the presence of gradient effects during the use of aminoglycoside antibiotics when incubated in the Tecan microplate readers. The location effect when using 96-well microplate readers has been observed in several other studies without the inclusion of antibiotics²⁸⁷⁻²⁸⁹, suggesting that the placement of a treatment on a 96-well plate influences result output regardless of antibiotic or bacterial inclusion. However, the gradient-effect in my data was most pronounced during use of aminoglycoside antibiotics. This observation could be due to differential oxygen gradients observed in the plate. Anoxic conditions have been shown to affect antibiotic susceptibilities, particularly aminoglycoside antibiotics, in four different bacterial species including *P. aeruginosa*²⁹⁰. However, more experiments need to be done to confirm this hypothesis.

Effects of multidrug therapy on bacterial extinction rate

Following evolution experiments, I first focused on evaluating the effect of each multidrug therapy on bacterial clearance. Extinction rate reveals the strength of the selective pressure imposed on the bacterial population and the probability of a resistance mutation arising (or pre-existing) to reach a high-enough frequency to be transferred to the next season. Cycling-in-combinations produced the highest number of bacterial extinctions in 4 out of the 5 evolution experiments, implying that this alternative therapy is comparatively difficult for populations to overcome (Fig. 9). Interestingly, in the single experiment that cycling drug combinations produced slightly lower clearance rates compared to the alternative therapies, cumulative growth of surviving replicates was correspondingly low (Fig. 9C). This result suggests that the genotypic or phenotypic change allowing these bacteria to avoid elimination had a detrimental effect on bacterial growth of surviving populations. Similarly, this was also the only experiment that generated a statistically significant fitness cost under drug-free conditions (Fig. 11). This further implies a substantial cost for populations able to overcome the initial extinction barrier, both in the presence and absence of antibiotics. Together, these results suggest that although populations exposed to cycling drug combinations did not yield the most clearance in this experiment, a larger cost is present for the surviving populations both during and after evolution, further providing evidence for the increased selective pressure present when cycling drug combination.

By comparing extinction rates between regimens containing the same antibiotics, important conclusions can be drawn about the type of drug application that is most challenging for the populations to overcome. For example, fluctuating environments have been shown to create a dynamic adaptive landscape to which bacteria struggle to adapt, as the continuous environmental changes complicate resistance evolution^{201,237,242}. Simultaneous addition of antibiotics similarly directly targets two metabolic processes concurrently, also creating a high selective pressure on bacterial populations⁵⁸. Cycling antibiotics^{201,237,239} and drug combinations¹⁹⁸ have both individually been linked to increased bacterial extinction compared to the conventional

monotherapy. However, studies that directly compare the potential of these two alternative therapies are rare, and results of said comparisons vary^{238,239}. For instance, Fuentes-Hernandez *et al*, identified cycling treatments that led to increased extinction rates in *E. coli* compared to the corresponding antibiotic combinations²³⁹. However, the aim of this study was to specifically identify cycling sequences that present increased extinction, and authors also state that several exceptions existed in which cycling drugs produced less extinction than the alternative combination treatments. Kim *et al* also evaluated the evolutionary outcomes of cycling versus combination therapy, but specifically focused on the rate of resistance acquisition. These experiments similarly found cycling therapy to be superior to drug combinations in select scenarios, but worse in others²³⁸. It is important to note that comparisons between hospital-wide combination (also known as mixing) and cycling protocols do exist, but will not be addressed in this discussion, as these alternative therapies aim to reduce the overall transmission of antibiotic resistance and not to delay resistance evolution within a single patient^{291,292}.

A similar extinction rate between cycling single drugs and drug combinations in 3 out of 5 experiments implies that cycling between and simultaneous addition of antibiotics generally does not significantly affect extinction outcome. Importantly, exceptions to this result do exist, as combinations belonging to the last two evolution experiment elicit no extinctions, while the same antibiotics used cyclically show an average of 20-40% extinction (Fig. 9D, E). The diverse extinction outcomes imply that additional factors other than the way in which the drugs are administered (regimen) affect extinction efficacy. Two additional factors that could influence extinction rate are the identity of the drugs applied and the physiological and evolutionary interactions of said drugs. Notably, no significant association was detected between drug interaction and extinction rate when testing drug combinations (Fig. S3). This result contradicts previous research which identified synergism¹⁹⁸ and the ability to evolve collateral sensitivity^{228,293} as a key factors in determining extinction rate in *P. aeruginosa*, *S. aureus*, and *E. coli*. One potential explanation for this discrepancy could be the limited number of tested combinations (two per experiment, as opposed to the 8 treatments in the cycling regimen), as well as the limited amount of combinations that display antagonistic interactions in PA (2 out of the 10 tested combinations interacted antagonistically, Fig. 5).

The underlying mechanisms behind antibiotic synergism and antagonism are not yet clear. It is known that physiological interaction cannot be obtained by simply grouping drugs by mode of action²⁹⁴, but instead appears to be specific both to individual drugs used and the species to which they are used against²⁹⁵. Physiological interactions have been found to be due to a physical interaction of drugs at their target, via a change in cell physiology which correspondingly affects the activity of the other drug, or simple uptake effects caused by one of the drug pairs, which secondarily increases uptake of the second drug²⁹⁵. For example, the synergy of antibiotics quinupristin and dalfopristin was recently shown to be due to an increased stabilization of the binding at the ribosome when both drugs bind²⁹⁶. The suppression detected when using colistin and vancomycin in combination in *S. aureus* was shown to be due to gene expression changes resulting from colistin which are similar to those in vancomycin-resistant mutants²⁹⁷. The synergy between aminoglycosides and β -lactams has been linked to uptake defects to the cell envelope caused by penicillin in *E. coli*, which increased uptake of streptomycin, thus promoting further cell damage²⁹⁸. However, it is important to note that this experiment was performed not by simultaneous antibiotic application, but by short drug pulses, and is therefore more likely to explain hysteresis effects than physiological interactions that may then also affect synergy. The wide

variation of known mechanisms behind physiological interactions additionally demonstrates that each interaction has an individual mechanism, and further research needs to be performed in order to understand the molecular basis of the interactions on a larger scale.

A significant association between both drug interactions (physiological and evolutionary interactions) and extinction rate was detected when testing populations exposed to cycling therapies (Fig. S4), suggesting presence of synergy and collateral sensitivity between the incumbent drug pairs increases extinction rates. Extinction rate has been seen to increase when cycling drugs displaying collateral sensitivity in *E. coli*^{217,239}. However, distinct differences in experimental setup from these experiments to my experiments should be noted. While my experiments involved fast switches, Imamovic *et al* selected for highly resistant clones over the course of several days, and following high resistance, antibiotics were changed²¹⁷. Fuentes-Hernandez *et al* cycled drugs irrespective of resistance profiles, but specifically selected the switching rate that showed the most extinction, which was not the treatment with daily antibiotic switches²³⁹. While scheduled cycling drugs displaying reciprocal collateral sensitivity have additionally shown to both decrease multidrug resistance as well as rate of adaptation^{238,243} in *S. aureus* and *E. coli*, the increase of extinction in response to collateral profiles during rapid, scheduled cycling therapy is, to my knowledge, a novel result.

Collateral profiles have been measured across many bacterial species, but the underlying mechanisms behind the profiles are not yet completely understood. Although it is clear that collateral sensitivity is the result of mutations acquired that confer resistance to one drug, that have a direct or indirect cost which alters susceptibility to the second drug, the molecular mechanisms behind each of these effects are not all currently known. However, certain mechanisms have nevertheless been researched in detail. For example, in *E. coli*, the aminoglycoside resistance induced reduced proton motive force, which consequently affected efflux pump functioning, creating hypersensitivity towards other antibiotics²⁹⁹. Collateral profiles can vary between bacterial species, and have been shown to differ between *E. coli* and in *P. aeruginosa*¹⁹⁹. Additionally, several studies have found that collateral profiles can vary, even between technical replicates in highly controlled *in vitro* experiments, caused by alternative paths to evolution of resistance^{199,207}.

Although individual antibiotics often have distinct molecular targets, drugs belonging to the same class can cluster both in the way they interact with other drugs and in terms of effectiveness^{198,300}. Summarizing extinction rates for all experiments by drug class highlights several relevant patterns (Fig. S12A). For drug combinations, the only antibiotic pairs that elicited any extinction in any of the tested experiments were penicillin+aminoglycoside combinations. This result is in agreement with previous publications that also found combining antibiotics from these drug classes elicited high extinction rates in PA14^{198,201}. The potency of penicillin and aminoglycoside combinations has been widely described³⁰¹⁻³⁰⁴, and combining drugs belonging to these classes is often the treatment of choice for many serious infections including endocarditis and prosthetic joint infections^{305,306}. The increased clearance rate seen when combining an aminoglycoside and a penicillin may stem from strong synergistic interactions often present when combining these two drug classes¹⁹⁸. The ability of many antibiotic pairs in these drug classes to evolve collateral sensitivity¹⁹⁹, compounded with the presence of negative hysteresis found to be present minimally between Gentamicin (aminoglycoside) and Carbenicillin (penicillin), could also contribute to the combination's strength. Drug cycles that include penicillins and

aminoglycosides receiving drugs cyclically also exhibit the highest amount of extinction in my data set (Fig. S12A), further substantiating the relationship between extinction and inclusion of antibiotics from these drug classes.

In addition to the importance on drug interaction and class on extinction rate, many populations which received the same antibiotics also showed prominent differences in extinction rate (Fig. 12), suggesting the importance of drug order on clearance rate. Differences in extinction rate for cycling-in-combination treatments were profound, with all experiments excluding one showing approximately two-fold differences in extinction rate depending on the order in which the antibiotics were applied (Fig. 12B, C, D, E). Discrepancies in clearance rate were similarly regularly seen for treatments within cycling regimens, with 10 antibiotic pairs showing 20-70% differences in extinction rate when the order of drugs is changed (Fig. 12, Table S11). The effect of drug order on resistance evolution has been seen for *P. aeruginosa* and *E. coli*^{201,237,239,249,307,308}. Two main hypotheses exist to explain this order effect, the first of which is the presence of asymmetrical collateral profiles. If the switch from antibiotic A to B results in collateral resistance, but the switch from B to A results in collateral sensitivity, one could assume that switching from B to A would result in more extinction, as the populations would find this switch more difficult to adapt to resulting from the collateral profile. Asymmetrical collateral profiles may be a potential explanation to the order effect in my data, as 5 pairs which showed more than 20% differences in extinction rate displayed asymmetrical collateral profiles, the majority of which were collateral resistance in one direction, and no significant collateral pattern in the remaining direction (Table S11). However, it is important to note that 3 drug pairs which showed large differences in extinction depending on drug order displayed symmetrical collateral profiles. Additionally, numerous pairs with asymmetrical profiles were seen in pairs that showed little to no difference, suggesting that this explanation was not the only predictor for the large differences in extinction shown by antibiotic order in this data set.

Hysteresis is an additional explanation that has been suggested to be responsible for the order-effect²⁰¹. Hysteresis states that the addition of one antibiotic simultaneously changes the cell physiology of the bacterial cell without changing the genotypic resistance profile. This physiological change subsequently affects how the cell responds to the second antibiotic in the cycling treatment. In experiments involving *P. aeruginosa*, switching from carbenicillin to gentamycin caused increased killing, while the reverse order caused increased protection²⁰¹. Differential killing capacities for these drug sequences depending on the drug order was also seen in my experiments. For instance, in experiment 4, starting with gentamycin elicited 25% extinction, while starting with carbenicillin yielded 100% extinction (Fig. 12D). Additionally, the majority of treatments composed of the same drugs applied in reverse order that also showed large differences in extinction rates were composed of an aminoglycoside and a penicillin. Within said aminoglycoside-penicillin pairs, the higher extinction rate was always achieved when the switch started first with a penicillin followed by an aminoglycoside (Table S11). The overall percentage of extinctions taken from all experiments in cycles starting with penicillins was ~65%, compared to ~35% when the cycle started with an aminoglycoside (Fig. S12). The mechanism behind this differential killing capacity depending on the drug class first deployed could be due to β -lactam antibiotics causing increased influx of the following aminoglycoside²⁹⁸, and conversely decreased killing could be due to an up-regulation of efflux pumps selected by aminoglycoside antibiotics^{121,309}. Together, these indicate that hysteresis is a viable

hypothesis for the phenomenon of divergent extinction rates seen when antibiotics are applied in opposing orders.

Growth yield changes in response to multidrug treatments

Next, I investigated the effects on overall growth inhibition of surviving populations in response to multidrug antibiotic therapies. Cumulative AUC measures all phases of the bacterial growth curve, first for each season, then total growth over all seasons, exhibiting how antibiotic therapies unable to eradicate bacteria inhibit growth of surviving populations. Bacterial load could also be seen as an additional population fitness measurement, estimating bacterial robustness in the presence of antibiotics and indirectly estimating bacterial resistance, as resistant cells behave as if the exposed drug was present in a lesser concentration⁵⁸. Growth dynamics were tightly monitored during evolution by measuring OD₆₀₀ values every 15 minutes and subsequently calculating the AUC inhibition relative to the evolving control, which serves as an additional parameter to measure rate of adaptation. Rate of adaptation monitors how quickly the populations are able to sidestep the growth defects normally observed in the presence of antibiotics. Both cumulative growth and rate of adaptation are important parameters in terms of understanding resistance evolution, as cells in a competitive environment that divide the quickest have an advantage over slowly dividing cells. Nevertheless, slow growth may be beneficial in antibiotic environments, as slow growth has been associated in many bacteria with increased bacterial tolerance to antibiotics³¹⁰⁻³¹². Slow growth has also been shown to provide temporary safe haven for bacteria that have not yet acquired resistance via two mechanisms. Firstly, slow growth increases the likelihood of resistance to emerge by upregulating mutation rates. Secondly, slow growth allows bacterial cells to bide their time, by increasing the time period during which the surviving populations can acquire the relevant resistance mutation(s) by chance¹⁰⁸. It is relevant to note that the 2% bottleneck imposed every evolutionary season may impose selection against slow growers, as these cells may not have enough time to replicate to a high enough extent to pass on material to the next season.

Cycling drug combinations generally yielded the lowest bacterial cumulative growth of all the alternative drug regimens in 3 out of 5 evolution experiments (Fig. 9B, C, E), suggesting this alternative therapy was not only able to elicit enhanced bacterial clearance, but additionally prolonged treatment efficacy. The prolonged inhibition in response to cycling antibiotic combinations in comparison to the alternative drug regimens may be due to the presence of 4 drugs over the course of treatment and the incumbent increase of physiological and evolutionary interactions present with multidrug therapies. However, it is important to note that not all experiments elicited significant differences in growth inhibition between cycling drug combinations and the remaining regimens, implying that increasing drug number was not always enough to delay evolution.

Cumulative growth in response to cycling drug combinations was significantly different from cycling single drugs in 2 experiments (Fig. 9B, E) and from combination therapy in 3 experiments (Fig. 9C, D, E). Importantly, the extent of the differences in growth inhibition were much larger when compared to combination therapy. For example, the cumulative AUC of combination regimens in evolution experiment 4 was approximately two-fold higher than that of cycling drug pairs (Fig. 9D), suggesting that either the addition of environmental heterogeneity, the addition of two additional antibiotics, or a combination of both aspects largely decreased bacterial adaptation rate. Although it is challenging to dissect which aspect more significantly contributes to

prolonged treatment efficacy, further comparing regimens provides valuable insight in understanding the success of this novel therapy. The gap between cumulative AUC of the two fluctuating environments was ~5 units in the most significant experiment (Fig. 9C), suggesting that the two heterogeneous environments performed similarly in prolonging bacterial inhibition compared to the static combination environment. Evolutionary dynamics similarly revealed populations adapting quicker and relatively low inhibitions as soon as seasons 3-4 when simultaneously exposed to 2 drugs (Fig. 10). I thus conclude that environmental fluctuation contributed significantly to therapeutic success in my experiments based on two main observations. Firstly, a similar bacterial load was produced when populations were exposed to fluctuating environments in the majority of experiments, regardless of the number of drugs included in the treatment protocol (Fig. 9A, C, D). Secondly, when comparing how all regimens inhibited growth, a comparatively small gap in bacterial loads was seen when relating the two fluctuating environments, even in experiments showing significant differences in overall growth (Fig. 9B, E). Additionally, although both regimens used only 2 antibiotics, simultaneous drug application produced higher cumulative AUC values in 4 out of 5 evolution experiments compared to cycling regimens, further proving that the fluctuation aspect of the treatment is seemingly crucial in delaying growth inhibition throughout evolution, though statistical significance was only present in 2 (Fig. 2D, E).

Temporal heterogeneity of antibiotic environments has been shown to decrease adaptation rate *in vitro* in *P. aeruginosa*^{201,237,249} and *E. coli*²³⁹, and has been further proven to decrease rate of multidrug resistance in *S. aureus*²³⁸ and *E. coli*^{240,243}. The benefit of fluctuating selection likely stems from a combination of factors including environmental switches interrupting selective sweeps and pleiotropic genetic interactions that occur when a population evolves resistance to one of the utilized drugs²⁴². However, it is crucial to understand that these environments have shown the potential to decrease rate of adaptation, and not every fluctuating environment was associated with decreased adaptation rate compared to static environments^{201,239,243,308}. My data is in agreement with these results as although cycling-in-combination regimens generally elicited prolonged treatment efficacy, it is relevant to note that substantial variation was present in treatments belonging to each drug regimen (Fig. 12). For example, though drug combinations largely inhibited bacteria the least throughout evolution, specific drug combinations, like gentamicin and carbenicillin, were very effective at prolonging growth inhibition, limiting growth as effectively as cycling drug combinations (Fig. 12B). Using this drug pair as an example, gentamicin and carbenicillin were successful regardless if applied simultaneously or cycled. This pair created a high number of extinctions in experiments 1 and 5 when cycled, while also effectively limiting growth of surviving populations. Similarly, this combination applied a high enough selective pressure to remove approximately 50% of the replicate populations when coadministered as a combination, while also significantly reducing overall growth. The efficacy of this particular drug pair is likely due, in part, to synergy¹⁹⁸, partial collateral sensitivity¹⁹⁹, and presence of hysteresis²⁰¹ previously demonstrated for this drug pair in *P. aeruginosa*. However, increased treatment efficacy in terms of high extinction rate and prolonged treatment success appears to be generally seen when combining an aminoglycoside and a penicillin. Regardless if drugs were cycled or simultaneously applied, combining these two drug classes always elicited the highest overall growth inhibition (Fig. S12B). More broadly, these results suggest that the efficacy of some combinations may be able to be generalized by antibiotic class, which would be helpful for clinicians to rationally design effective therapies.

The success of multidrug treatment has been linked to the incumbent drug interactions, both physiological and evolutionary⁵⁸. I therefore analyzed the impact of drug interaction (FCR and PIS) on overall growth inhibition individually for cycling and combination regimens. A significant association both between drug interaction (both FCR and PIS) and growth inhibition was found for both cycling and combination regimens (Fig. S3-4, Table S14). Meaning that synergistic interactions were more likely to limit bacterial evolution in both cycling and in combination environments. Correspondingly, pairs displaying collateral sensitivity were also more likely to show prolonged treatment efficacy in both cycling and combination environments. To explore these associations, I will first focus on combinations and the incumbent drug interactions contributing to decreased overall bacterial growth.

The effects of deploying synergistic antibiotic combinations are debated. Many experiments have provided evidence that higher initial efficacy and higher selective pressure translates into later increased prevalence of drug resistance and/or adaptation rate in *E. coli*^{214,222,223} and *S. aureus*²²⁵, although synergistic antibiotics are still combinations of choice for clinical application^{313,314}. My experiments gave contrasting results, as synergistic combinations not only yielded the highest clearance rates, but additionally were also significantly correlated to high inhibition throughout evolution (Fig. S3B,D). Evolutionary dynamics confirm that experiments containing antagonistic interactions were the only experiments yielding visible differences in adaptation, with the combination treatments adapting much faster relative to the remaining therapies, though experiment 3 also showed phases where combinations adapted quicker (Fig. 10C, D, E). The differences between my work and the previously described experiments could stem from one of several reasons. Firstly, the utilized drug concentration has been shown to have a large effect on the evolutionary outcome²⁴¹. While many previous experiments tested gradients of antibiotics, and specifically chose populations which were able to grow at high drug concentrations, I included only a single concentration which consisted of serial dilutions of the component antibiotics and translated to an initial inhibition of 80%^{214,223,225}. Secondly, resistance values are not known for the combination pairs showing synergistic interactions. One of the mentioned experiments exclusively tested the effects of synergistic combination on the evolution of single and multidrug resistance³¹⁵. It is therefore possible that although synergism increased inhibition over the length of evolution, it also selected for increased single or multidrug resistance. Lastly, previous studies compare the effects of 2-3 drug pairs in order to generate conclusions^{222,223,315}. This low inclusion number could bias the results and conclusions drawn from such data sets, as my data emphasizes substantial variability can arise depending on the included antibiotics.

Evolutionary interactions have also been shown to affect evolutionary consequences of drug combinations applied simultaneously *P. aeruginosa*¹⁹⁸, *S. aureus*²²⁸ and in *E. coli*^{227,316}, namely that the probability of collateral sensitivity to evolve in a drug pair decelerates resistance evolution. My data also demonstrated a significant association between probability to evolve collateral sensitivity and increased growth inhibition throughout evolution (Fig. S3B, Table S14). These results emphasize the importance of researching both physiological and evolutionary interactions and highlight a potential interconnection between these two predictors.

The success of cycling treatment was also found to be associated with both evolutionary and physiological interactions (Fig. S4B, D), with drug pairs exhibiting collateral sensitivity and/or synergism yielding lower overall bacterial growth than collaterally resistant pairs. Correlations between evolutionary interaction and rate of adaptation during cycling antibiotic therapy have been identified in *E. coli*^{239,240}.

However, experimental setups varied greatly in said experiments. One experiment specifically looked to see if drug pairs displaying collateral sensitivity could decrease rate of adaptation, and thus subjected bacteria to high drug concentrations to first achieve resistance, and only when a certain level of resistance was seen were the bacteria switched to the second drug²⁴⁰. A second experiment was focused on the eradication of bacteria under low doses of cycled drugs, and identified several multidrug regimens with different switching rates that decreased rate of adaptation²³⁹. The interaction between overall bacterial load as a proxy for rate of adaptation and the presence of collateral sensitivity and synergism in fast-switching cycling drug regimens is, to my knowledge, a novel finding.

It should be noted that the presence of an association between drug interaction and bacterial load does not mean that drug interaction is the sole explanation for treatment efficacy in terms of growth inhibition. Exceptions in the data do exist, and there are several cases in my data in which combinations or cycling treatments that display vastly different PIS or FCR scores elicit similar growth inhibitions (Fig. S3, Fig. S4). This result implies that additional factors affect limitation of bacterial growth during antibiotic treatment, one of them likely being the presence of positive or negative hysteresis²⁴². Additional factors including but not limited to treatment duration and the switching rate³¹⁷, population size and structure²⁰⁸, resource limitation leading to competition and cooperation³¹⁸, an increased mutation rate in response to the presence of select drugs³¹⁹, drug concentration³²⁰, and physiological drug interaction³¹⁵ also presumably affect the success of both cycling and simultaneous drug application.

Bacterial clearance rate and overall growth inhibition of surviving populations predict differential aspects of antibiotic treatment successes. Comparing between measured phenotypes helps to further understand how said estimators overlap or differ. Significant negative correlations between extinction rate and cumulative growth were identified in 3 evolution experiments (Table S7), demonstrating that populations exposed to relatively high selective pressure (and thus showing high extinction rates) were generally more inhibited by the remaining drug treatment. This data contradicts previous studies that argued an increased initial selective pressure speeds up evolution and leads to decreased long-term bacterial clearance²²². However, it is important to note that correlations varied in strengths, and that 2 experiments did not show significant correlations, implying that additional parameters (likely physiological and evolutionary interactions of tested drugs, as discussed above) influence these measured phenotypes.

Fitness costs in response to multidrug environments

I continued to examine bacterial responses to multidrug environments by assessing fitness costs in surviving populations. The ability to evolve resistance to antibiotics is frequently associated with a fitness cost⁷⁴. Understanding how different therapies promote fitness costs is an important part of assessing therapeutic efficacy, as the cost of resistance is one critical in determining the rate and extent of resistance emergence^{71,72,74}. In clinical cases of infection, bacteria causing disease generally do not exist alone, but instead exist in bacterial communities³²¹. Therefore, a mutation which renders the cell in some way less fit, decreases the ability of the cell to survive and multiply in a drug-free environment, and is thus likely to be outcompeted by the remaining, sensitive surrounding cells⁶³. Additionally, fitness costs could be exploited to select for the highest cost which has the lowest chances of propagation, thus limiting resistance transmission. A bacterial fitness defect can be reflected in a reduced competitive ability³²², growth defect³²³, or reduced virulence³²⁴. My experiments used

growth curves in the absence of antibiotics to determine potential fitness costs in response to antibiotic resistance.

I found that most populations showed growth defects relative to the evolving control, indicating that withstanding these harsh environments was associated with a corresponding cost in terms of growth in no-drug media (Fig. 11, Fig. S5, Fig. S6). However, occasionally in the data set, certain lineages showed no detectable fitness defects, or even increased fitness relative to the evolving control. While growth defects are commonly seen in response to antibiotic treatments, the lack of or increase in fitness could be explained in three ways. First, compensatory mutations can ameliorate fitness costs through a secondary mutation, and are regularly observed in drug-resistant bacteria⁷⁶. Compensatory mutations can neutralize the fitness cost of resistance mutations by restoring the structure or function of the previously deleted RNA molecules, proteins, or other damaged cellular machinery; or by simply bypassing the mutated function and replacing it with an alternative pathway⁷⁶. Compensatory mutations have been shown to either neutralize or increase the fitness of resistant bacteria in *Neisseria gonorrhoeae*³²⁵, *Mycobacterium tuberculosis*³²⁶, and *S. aureus*³²⁷. Despite the general association between evolved resistance and fitness costs, additional evidence has shown that some mutations do not appear to cause any measurable fitness cost in the first place^{328,329}. A second explanation for the absence of a fitness cost in select replicate populations could therefore be that a resistance mutation was indeed present, but either the costs were not detected via growth in no-drug media, or said mutation simply was not associated with measurable fitness deficiencies.

A third explanation for the neutral or increased growth seen in some replicates could be a general lack of genetically encoded resistance. Although the bacteria showing relatively little growth inhibition in high drug concentrations are expected to be resistant following 7-day antibiotic treatment, persistence and/or tolerance could additionally explain the lack of resistance costs. Bacterial persistence has been defined as the ability of a subpopulation of bacteria to survive exposure to bactericidal drug concentrations through metabolic dormancy³³⁰. Tolerant bacteria are a population of cells able to transiently survive high concentrations of antibiotics without an associated increase in the MIC³³⁰. Although not yet fully understood, experimental evidence has shown that persistent and tolerant bacteria survive antibiotic treatment through metabolic dormancy and reduced ATP levels³³¹. Persistence has additionally been described for every tested bacterial species, and has been associated with treatment failure for many infection-causing organisms^{332–335}. Despite not being directly assayed in my experiments, I saw several bacterial replicates which showed the potential for tolerance and/or persistence, as the population was inhibited fully throughout evolution, but as soon as no-drug media was applied, the population spiked and grew as well as the evolving control (Fig. 14D: S~Ca, A~S, E: S~P, D~Ci). Further experiments need to be performed in order to confirm this hypothesis, but nevertheless, persistent and/or tolerant growth are probable mechanisms that would allow bacteria to survive exposure to antibiotics, without an associated fitness cost.

By comparing fitness costs among regimens, one should expect to learn more about the selective pressure imposed by each therapy. Strength of selective pressure has been shown to influence evolutionary outcomes^{241,336}, meaning that different selective pressures steer evolution towards different evolutionary paths with different corresponding fitness costs. For example, weaker selective pressures lead to step-wise mutations which select for lower fitness costs³³⁷. Interestingly, the majority of experiments elicited no significant differences in fitness costs with the exception of experiment 3, in which cycling drug combinations produced lower maximum yields

than the remaining therapies (Fig. 11). The similarities in fitness costs suggests that although these therapies lead to different extinction rates and growth yields, the corresponding fitness costs are relatively similar. Similar fitness costs present in regimens with theoretically different selective pressures (as a consequence of the higher number of drugs used in cycling therapy or the presence of synergism/collateral sensitivity between select drugs) could be explained by the fact that each tested multidrug therapy is arguably a high selective pressure in itself. For example, the use of two or more antibiotics has been shown to select for broad-spectrum resistance mechanisms like increased efflux expression in PA³³⁸. The selection of more general resistance mutations which allow bacteria to survive better in multidrug environments could explain the similarities between fitness costs for all tested regimens.

Similar to bacterial extinction rate and growth yields, fitness costs varied significantly depending on the specific antibiotic applied (Fig. 13). However, unlike the previously discussed phenotypes, no associations were found based on physiological or evolutionary interaction (Fig. S7). As the presence of synergism or collateral sensitivity arguably increases the selective pressure on a bacterial population, these results further confirm that selective pressure had no measurable influence on fitness defects in my data. Furthermore, no measurable correlation was found between relative maximum yield and extinction rate or growth inhibition throughout evolution, again reinforcing the hypothesis that selective pressure does not affect fitness costs as measured by growth in no-drug media (Table S7). Altogether, my data reveals no measurable patterns in fitness costs associated with regimen nor with antibiotic interaction, suggesting that fitness costs are similar in each of the multidrug treatments.

Replicates respond differentially to multidrug environments

Thus far I have provided evidence that cycling drug combinations has the potential to increase bacterial clearance rates while prolonging treatment efficacy, without any measurable decreases in bacterial fitness. However, as discussed above, substantial variation within the regimens was also observed depending on the chosen drugs, physiological and evolutionary interactions, and antibiotic class. I further detected variation in replicates being exposed to the same environment in overall growth yield, evolutionary dynamics, and fitness costs. For example, evolutionary dynamics in terms of AUC inhibition of individual populations show replicate populations were not adapting to the multidrug environments at the same rate (Fig 14). For instance, cycling between S~D in experiment 5 revealed several replicates fairly quickly and uniformly adapting to the multidrug environment, with inhibitions around seasons 4-5 significantly decreasing to ~50% (Fig. 14E). Notably, 3-4 replicate populations did not adapt so easily, and seemed to find their own paths to adaptation during later seasons. This sort of divergent evolution was found in every evolution experiment and was largely prominent in the fluctuating environments (Fig. 14). Interestingly, two combination environments (S+Ca, G+Ca, Fig. 14A, B) also produced divergent evolution, likely because the addition of a penicillin and aminoglycoside created a more difficult adaptive landscape for the bacteria to overcome, even in a static environment.

Although evidence has shown rational design of antibiotic therapies based on the evolutionary interactions of utilized drugs has the potential to decrease resistance evolution and limit multidrug resistance^{217,299,300,339,340}, these experiments have been performed with a limited amount of replication. Increasing the number of tested replicates has identified divergent evolution in individual bacteria populations

responding specifically to fluctuating antibiotic environments in *P. aeruginosa*¹⁹⁹, *S. aureus*³⁴¹, and *E. coli*^{207,241}. This divergent evolution reveals stark differences in evolutionary responses to secondary antibiotics including where some replicates evolved collateral resistance to a primary drug, while the remaining replicates exhibited collateral sensitivity^{199,207}. Distinct genotypic mutations underlie the phenotypic differences in resistance^{199,207}, further highlighting the various adaptive paths possessed by bacteria to achieve resistance. My data is in agreement with these studies, as replicate populations exposed to identical antibiotic environments often adapt at different rates and degrees and exhibit unique fitness costs (Fig. 13, Fig. 14).

It is important to note that not all environments promoted divergent evolution, as some replicates behaved similarly in multidrug environments (Fig. 14A, G~S; Fig. 14E, Ci~D; Fig. 14A, G+P, S+Ca). This result suggests that certain multidrug environments promote divergent bacterial responses, perhaps stemming from a higher selective pressure in which one simple path towards evolution is not always evident. Increased prevalence of divergent evolution was seen in fluctuating environments, particularly when cycling drug combinations and when including a penicillin and an aminoglycoside in the multidrug treatment sequence. As previously discussed, penicillins and aminoglycosides used together exhibit a high amount of bacterial clearance and high bacterial inhibition throughout evolution regardless of the way in which the drugs are applied (Fig. 12). I thus propose increased selective pressure, likely resulting from presence of synergy and reciprocal collateral sensitivity exhibited by penicillin and aminoglycoside antibiotics, as a potential selector for divergent evolution in my experiments. Strength of selective pressure has been determined to be an important predictor of evolutionary complexity, and higher selective pressures have led to increased genomic complexity in *E. coli*²⁴¹. The influence of selective pressure on divergent evolution, however, needs to be further studied in order to identify concrete relationships.

Resistance phenotypes in response to multidrug environments

As divergent phenotypic responses to multidrug environments were common on my data, I sought to investigate if these differentially performing replicates had correspondingly distinct resistance profiles. Following evolution, I assessed phenotypic resistance of selected populations from evolution experiment 5. Resistance changes are a fundamental aspect for determining antibiotic treatment efficacy, as a primary goal of multidrug therapy is to decrease the rate of single and multidrug resistance. Selecting against multidrug resistance is of particular importance, as resistance to more than one drug has been repeatedly linked to increased mortality in patients suffering from bacterial infections³⁴²⁻³⁴⁴. Increasing drug number as a means of decreasing multidrug resistance has been criticized, as engaging multiple bacterial targets can theoretically select for more broad-spectrum resistance mechanisms and has been shown to increase expression of multidrug efflux pumps and thus multidrug resistance in *P. aeruginosa*³³⁸. In contrast, however, antibiotics that engage multiple bacterial targets have contrastingly been shown to be more successful than drugs targeting single processes^{345,346}, and as discussed in detail above, the success of multidrug treatments depends largely on the individual antibiotics used and their respective physiological and evolutionary interactions. Notably, changes in resistance are often primarily associated with resistance increases, however increases in sensitivity are also frequently observed^{217,228,299}. Increased drug susceptibility is an equally important phenomenon as it can be used to steer resistant populations and deter evolution of multidrug resistance.

I found both increased resistance and increased sensitivity in every tested treatment and in both analyzed timepoints (Fig. 17). The majority of populations increased their resistance to at least one of the utilized drugs, suggesting that chromosomal mutations were frequent. In general, resistance in all multidrug environments evolved to a part of the antibiotic treatment, commonly producing either neutrality or increased sensitivity to the remaining utilized antibiotics. This finding suggests that multidrug treatment does not always select for multidrug resistance, though in order to fully make this assumption, evolution of single drug therapies would need to be included in my experimental design. Notably, multidrug resistance was present in my data, though interestingly was not more prevalent in cycling drug combinations which included 4 instead of 2 antibiotics (Fig. 18A). This finding demonstrates that adding additional antibiotics does not associate with increased multidrug resistance. Antibiotic exposure, similar to any selective pressure, inarguably selects for resistance. However, when the drug concentration is kept constant, utilizing 4 drugs appears to select no more for multidrug resistance than deploying 2 drugs in my experiments.

Surprisingly, cycling single drugs produced a significantly higher MDR index than cycling between or constant drug combinations (Fig. 18A). This result is contradictory to previous findings which showed that applying drugs cyclically constrains multidrug resistance evolution compared to monotherapy and simultaneous drug application²³⁸. However, the increased multidrug resistance in cycling treatments in my experiments appears to be due, in large part, to outliers which show high amounts of multidrug resistance, potentially biased by the high levels of resistance elicited to streptomycin and ciprofloxacin in cycling treatments (Fig. 18B, Fig. 17). Nevertheless, the increased multidrug resistance identified in cycling single drugs highlights the importance of testing many different drugs, and implies that cyclical drug application increases multidrug resistance more so than the alternative tested regimens.

The impact of evolutionary and physiological interactions appears to be minimal on resistance profiles in my data. For example, ciprofloxacin and doripenem display partial collateral resistance, and populations exposed to this drug pair show substantially less multidrug resistance than those exposed to streptomycin and doripenem, which exhibit partial collateral sensitivity (Fig. 5, Fig. 17). Additionally, the combination ciprofloxacin and streptomycin share an antagonistic relationship and generate high levels of single and multidrug resistance, while doripenem and piperacillin/tazobactam interact additively and display almost no resistance at either tested timepoint (Fig. 5, Fig. 17). Resistance due to simultaneous application of drugs has been shown to be suppressed when using collaterally-sensitive drug pairs³¹⁶. I, however, saw no evidence of this in my data, as ciprofloxacin and streptomycin displayed partial collateral sensitivity and showed an abundance of single and multidrug resistance while doripenem and piperacillin/tazobactam showed partial collateral resistance and elicited virtually no resistance (Fig. 17, Fig. 18). The inconsistencies between these results could be due to a variation in direction and extent of measured interactions between drugs depending on the evolutionary path taken and selected for by the population^{199,207}. Additionally, the sample size included in the characterization analyses may need to include more treatment types in order to further generalize.

Interestingly, populations displaying no changes, or even slightly enhanced susceptibility to utilized drugs, were also identified following resistance measurements, specifically during earlier seasons of evolution. This result demonstrates that bacterial subpopulations or entire bacterial populations were able to survive in multidrug

environments without a corresponding change in resistance levels, implying that tolerance and/or persistence was present in my experiments. Increased resistance present in isolates measured after 14 seasons of evolution implies the phenotypic change is transient and the longer the cells are kept in antibiotics, the more resistance is selected, though the increased resistance seen in late seasons was not always significant. A synergistic interaction between tolerance, persistence, and resistance has been identified in *P. aeruginosa*³⁴⁷ and in *E. coli*³⁴⁸, meaning that cells that show these non-genetically encoded phenotypes are also more likely to become resistant. Additional experiments have identified persistence¹⁰⁸ and tolerance¹⁰³ as able to promote the evolution of antibiotic resistance by increasing the time during which they are able to acquire genetic resistance. My data also fits with these findings, as the absence of resistance was more prevalent in isolates from season 7 than season 14.

An absence of increased resistance was extensively present in cycling-in-combination treatments during season 7, but was also seen in certain populations exposed to combination and cycling single drugs (Fig. 17). One particularly striking example was the simultaneous application of piperacillin/tazobactam and doripenem, where no increases in resistance were identified, even after evolving in this drug combination for 14 seasons (Fig. 17B). Interestingly, populations exposed to this treatment exhibited relatively little growth inhibition (Fig. S8), zero extinctions (Fig. 12E), and only a mild decrease in fitness measured in no-drug media (Fig. 13E). Notably, when fitness of individual colonies was measured, these clones were one of the few isolates showing a slight increase in time spent in lag phase. The extension of lag phase as a mechanism mediating antibiotic tolerance without resistance acquisition has been shown in *E. coli*³¹². Additionally, resistance to the antibiotic tetracycline and quinolone antibiotics was most often attained via extension of lag phase in *P. aeruginosa*³⁴⁹. These findings offer a possible explanation as to the phenomenon of prolonged antibiotic susceptibility seen in my dataset; however, more experiments are needed in order to confirm the presence of persistent or tolerant phenotypes.

Heterogeneity within a single population exposes presence of bacterial phenotypic subpopulations

Heterogeneity was ubiquitous in my data set, highlighting the extreme adaptability of *P. aeruginosa*. I therefore continued the analysis by investigating an additional layer of heterogeneity in the data and tested for differences in phenotypic resistance patterns observed in single colonies isolated from a single evolving population. As outlined above, replicates exposed to the same drug treatment often showed differing resistance profiles. These results are in agreement with several recent publications reporting divergent collateral responses to a second drug through differing paths of chromosomal resistance^{199,207,241,341}. Such examples of divergent evolution can bolster fitness and probability for survival during antibiotic treatment, as increased population heterogeneity corresponds to increased likelihood that select individuals are able to adapt to fluctuating environments^{350–352}. Measuring phenotypic and genotypic variance in response to antibiotics is essential to further understanding bacterial resistance evolution and to designing customized antibiotic therapy. Only when all possible evolutionary paths are taken into account, in addition to controlling for the complex and stochastic processes that influence the chosen path, can rational antibiotic treatment be effective in controlling bacterial evolution. Therefore, not only variation in technical replicates, but variation within a single evolving population is also fundamental to understanding the consequences of multidrug therapy.

I found the presence of phenotypic heterogeneity in every tested treatment type (Fig. 16A), revealing that subpopulations within a single evolving population exhibited differential resistance levels even in this well-mixed population extremely unstructured environment. While the presence of subpopulations expressing differential resistances to tested antibiotics could and likely does stem from a combination of genetic and non-genetic mechanisms, I will focus on non-genetic mechanisms of phenotypic heterogeneity for two main reasons. Firstly, genomes of evolved isolates are not available for this data set, and therefore it is difficult to draw any concrete conclusions about chromosomal mutations. Secondly, while most genotypic mutations impose a measurable fitness costs⁶³, I saw no evidence of a correlation between extent of single (Fig. S10) or multidrug resistance (Fig. S11) and fitness costs. Furthermore, no distinct patterns were seen when grouping members of a subpopulation and the extent of similar fitness costs. Though the possibility of genetic variations in a single population cannot be ruled out, I have no evidence to support this hypothesis. Additionally, if the subpopulations were due to resistance mutations, the fitness of the subpopulations should cluster together, but this is not the case. It is, however, important to note that compensatory mutations could mask fitness costs, concealing evidence for similar fitness costs brought about by genetic mutations underlying phenotypic differences in the data.

Phenotypic variation is prominent in structured bacterial environments including biofilms^{353,354}, as switching from planktonic to biofilm growth requires genetically identical cells to drastically alter motility, antibiotic tolerance, transcription expression profiles, and polysaccharide production³⁵⁵. Astoundingly, not only do cells switching from free-swimming to biofilm growth behave differently, but cells within the biofilm drastically differ, with in proteome production differences reaching up to 50% in *P. aeruginosa*^{356,357}. In addition to heterogeneity in biofilm-forming cells, phenotypic heterogeneity has also been observed in planktonic cells, with differences in metabolic activity³⁵⁸, growth rate³⁵⁹, compound secretion³⁶⁰, virulence³⁶¹, and resistance to stress³⁶² reported in clonal, planktonic cell culture. Heteroresistance is another example of subpopulation-mediated resistance in which a subpopulation of isogenic bacteria are able not only to survive, but to replicate rapidly in the presence of otherwise toxic antibiotic pressures³⁶³. Phenotypic heterogeneity can arise from environmental cues or stressors^{364,365} or stochastically^{366,367}, and in either case shows phenotypic variation in even genotypically-identical cells. While further experiments need to be performed in order to distinguish the mechanism underlying the phenotypic variation in my data, both genotypic and phenotypic could explain this result.

Variation in phenotypic resistance was not uniform in all tested drug treatments and regimens, suggesting that particular treatment types selected more for divergent phenotypes than others. For example, the number of subpopulations, and thus diversity in each well, was generally higher in fluctuating environments than in stable, combination environments (Fig. 16). Both fluctuating environments routinely generated 3 diverging subpopulations, while simultaneous drug application produced at most 2 subpopulations, and many treatments exhibited only a single, phenotypically similar population. Fluctuating environments have consistently been linked to population heterogeneity over a range of organisms and species, as the population hedges their bets to increase probability of survival^{362,368-370}. Alternatively, the increase in population diversity could be due to differential selective pressures present in each season, which favor distinct genotypes, but are not present for a long enough time span for the mutation to fixate within a population. Interestingly, the increase in diversity in response to fluctuating environments only was statistically significant when cycling

drug combinations, suggesting that the increased environmental complexity in addition to fluctuation specifically seemed to select for population diversity (Fig. 16B). Though the number of subpopulations inferred from both fluctuating environments was generally higher than those seen in static, combination environments, the additional 2 drugs introduced in the cycling-in-combination treatments appears to somehow specifically select for increased population diversity.

Increased diversity in response to particularly cycling drug combinations is an important observation in terms of treatment efficacy, as the increased bacterial survival seen resulting from increasing population diversity has been linked to antibiotic treatment failure. For example, the presence of subpopulations with increased resistance to utilized drugs mediates treatment failure in *Enterobacter cloacae*³⁷¹. Heteroresistance in *S. aureus* has also been linked to vancomycin treatment failure³⁷². Diversity caused by subpopulations of persister cells has been shown to lead to treatment failure in a variety of bacterial species^{334,335,373}. Therefore, although cycling drug combinations appears to be superior to the alternative tested regimens in terms of extinction, prolonged inhibition, and multidrug resistance, caution needs to be exercised when drawing too general conclusions about the efficacy of this novel therapy. The increase in phenotypic heterogeneity in combination with a lack of significantly higher fitness costs presents could argue against the use of this alternative regimen, as the resulting divergent population could theoretically be more likely survive additional antibiotic pressures.

The influence of time spent in evolution on population heterogeneity provided contrasting results. While no major changes in abundance of subpopulations was generally identified by comparing the two tested evolutionary time points (Fig. 16), differences in subpopulation number in certain populations at two tested time points suggests that the time spent in evolution may have an impact on population diversity. However, my results did not provide evidence for increased time in evolution selecting for either increased or decreased population diversity. This result rather argues against the presence of bet-hedging in my data, as if bet-hedging were present, more diverse populations would be selected for with increasing time spent in evolution. Instead, the amount of diversity seemed to be dependent on both the component regimen and the individual population. For example, cycling drugs in combination may select for decreased variation over time, as two populations which showed differences in population diversity decreased in number of subpopulations during later evolutionary time points. In contrast, the single population in drug combination environments that showed differences in subpopulation abundance over time showed an increase in subpopulations during later time points. Interestingly, populations exposed to cycling single drugs showed both decreased and increased heterogeneity throughout evolution, even when exposed to the same drugs. In order to draw more concrete conclusions about the influence of time spent in evolution on phenotypic variability, more populations need to be included in the analysis and the gap between testing points most likely needs to be increased.

Fitness costs are not related to presence nor extent of drug resistance

The impact of increased resistance on bacterial growth is a fundamental aspect of antibiotic treatment efficacy, as fitness costs can be exploited in different environments and outcompeted by sensitive cells. While fitness costs were roughly measured for all populations following evolution, measuring specific colonies in which resistance patterns were also measured allows direct comparison between single and

multidrug resistance and associated fitness costs. Similar to population fitness measurements, I found that colonies generally exhibited a decrease in fitness relative to the ancestor PA14 strain. However, importantly, fitness benefits present in select colonies were occasionally seen throughout the data. The associated costs similarly differed depending on the assayed colony, with variation in costs being ubiquitous for colonies in each well (Fig. 19). Although resistances to select antibiotics were weakly correlated to decreases in fitness (Fig. S10), no strong correlations were identified between presence or extent of single or multidrug resistance and resistance costs (Fig. S10, Fig. S11).

The lack of correlations between resistance and fitness costs could be explained in one of three ways. Firstly, population genomes are not available and thus, genetic resistance mutations, although likely, are not confirmed. If the differences in resistance measurements were influenced by phenotypic resistance heterogeneities like heteroreistance, persistence, or tolerance, this could bias the correlations as many phenotypic resistance mechanisms do not display measurable fitness costs in the absence of antibiotics^{104,348,374}. Secondly, as fitness costs were only measured following evolution, this is likely enough time for compensatory mutations to arise, as these can take as little as 5 days to emerge^{325,375,376}. Lastly, although the majority of resistance mutations conferred associated growth costs, notable exceptions existed that carried no measurable costs^{63,377}. Each of these hypotheses likely contributes to the absence of strong correlation between resistance and fitness costs in my data.

Conclusions

The insights from this thesis provide information to further understanding evolutionary dynamics in response to multidrug environments. I present cycling drugs in combination as a potential novel alternative antibiotic therapy that causes increased extinction rate and prolonged treatment efficacy compared to cycling single drugs or simultaneous application of two antibiotics. However, I advise caution when drawing such clear-cut conclusions, as exceptions were plentiful in this data set. For example, increased presence of subpopulations was also detected in populations undergoing drug combinations applied cyclically, and population diversity has mediated treatment failure in several model organisms^{363,371}. It is additionally important to note that although cycling drug combinations were generally very successful, certain populations exposed to cycling single drugs or simultaneous application of two drugs also elicited high extinctions and prolonged growth inhibition. These results highlight that the equivocal responses of bacteria to multidrug environment are dependent on auxiliary factors and are influenced by more than simply the applied drug regimen.

I additionally want to emphasize that these results are specific to PA14, our experimental setup, utilized drugs, time between switches, and to the applied selective pressure (IC_{80}), and results would likely differ if any one of these aspects were altered. Additionally, these experiments do not include the complexity of an *in vivo* environment, where infections are likely composed of many competing species, environments are highly structured, nutrition is limited, and immune cells are constantly present. Nevertheless, evolutionary outcomes in highly controllable, specific environments provide fundamental information to further understanding how antibiotics steer evolutionary outcomes, which I argue is a cornerstone to designing rational drug treatment strategies.

I do not wish to imply these results will provide a solution to the antibiotic crisis, as there is likely not one superior treatment which will solve this problem. But rather I want to emphasize that the solution to this global problem lies in understanding the stochastic and predictable evolutionary processes brought about by drug treatment. With cycling drug combinations, I offer a way to prolong the shelf-life of currently effective antibiotics by applying said drugs in temporally differential manners. Resolving this global problem will require an interdisciplinary effort involving combining data from *in vitro*, *in vivo*, and clinical data to fully understand the complexity that is bacterial evolution.

Supplementary Information

Supplementary Figures

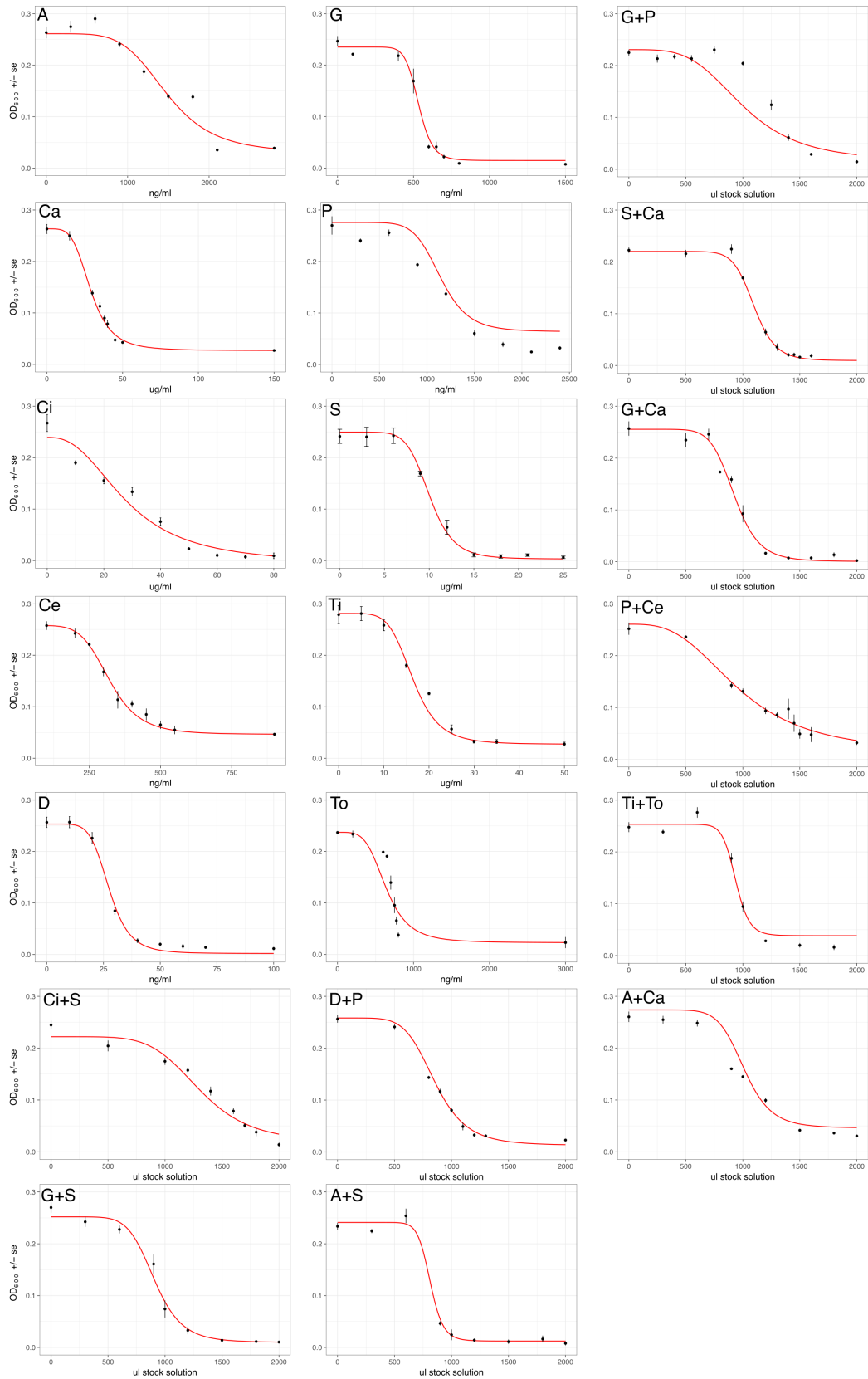


Figure S1 Dose response curves of single drugs and antibiotic combinations. Prior to each evolution experiment, dose response curves were run for both combination and single drugs in order to accurately estimate the drug concentration that achieved a bacterial inhibition of 80%. Isogenic bacteria were grown in M9 minimal media for 5-7 hours at 37°C in order to achieve a mid-exponential phase bacterial culture with a corresponding OD₆₀₀ of 0.08. During incubation, 10-20 antibiotic dilutions were prepared and pipetted into the 96-well plates in a block-randomized design. Approximately 10⁵ bacterial cells in mid-exponential phase were inoculated into each well containing differing concentrations of antibiotics. The plate was then shaken in Tecan plate reader machines for 12 hours at 37°C, and OD₆₀₀ was measured after incubation.

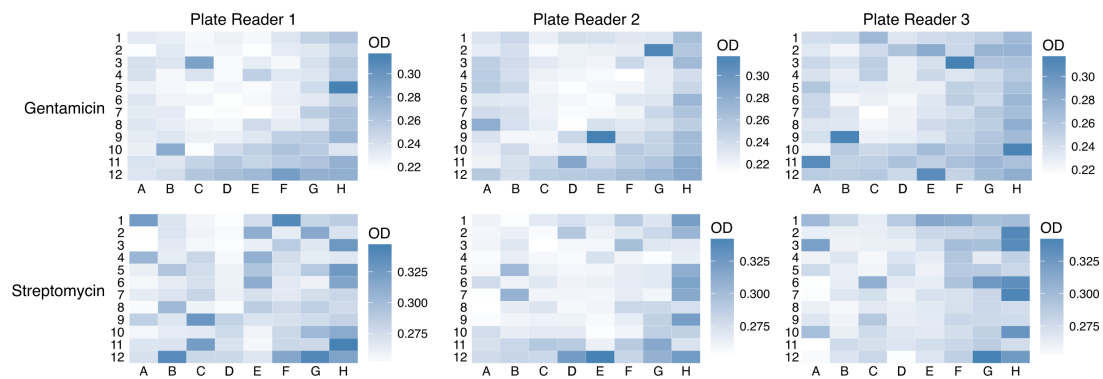


Figure S2 Gradients in plate reading machines with aminoglycoside antibiotics. In response to the variation present in dose response curves, presence of gradients in the plate readers to be used during the experiment was assessed. IC₂₀ concentrations of antibiotics were incubated with bacteria in 96-well plates for 24 hours at 37°C shaking. After 24 hours, OD₆₀₀ was read, and data was analyzed using the ‘ggplot2’ package in R to assign gradient colors to each well³⁷⁸.

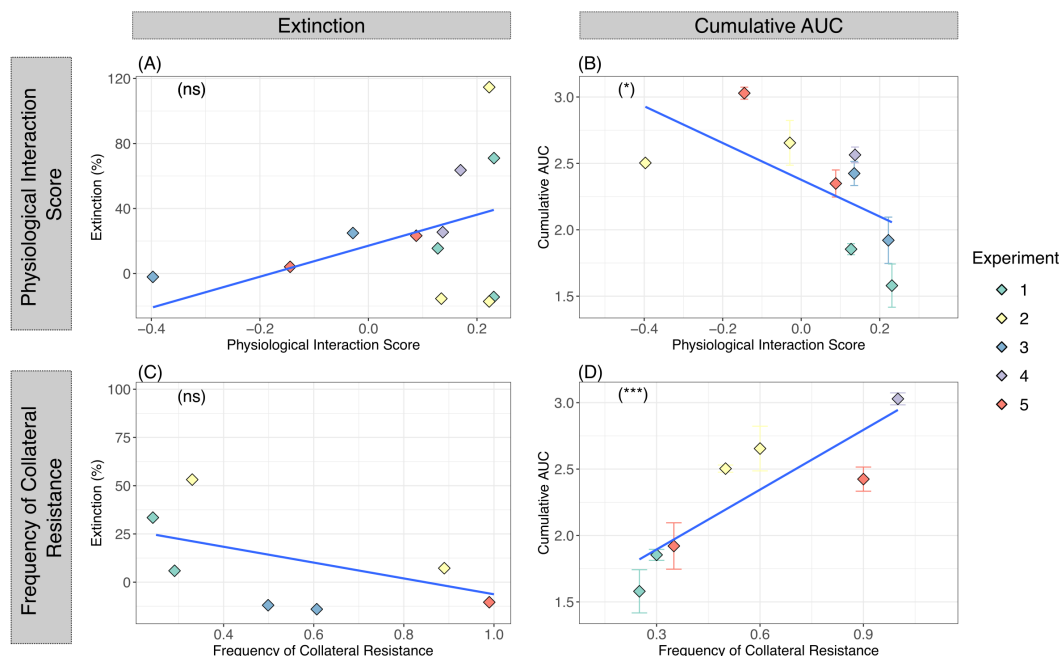


Figure S3 Associations between the applied drug interactions (both physiological and evolutionary interactions) and treatment success (extinction rate and cumulative AUC) when applying drug combinations. Physiological and evolutionary interactions were previously measured for the utilized PA14 ancestor^{198,199} (see Materials and Methods). Panels (A) and (C) show how the physiological interaction score (PIS) and frequency of collateral resistance (FCR) influenced extinction rates. All treatments included in the combination regimen for which

the PIS and FCR scores were available (n=8-12 replicates per treatment, n = 2 treatments per experiment) were taken from the data. Each point represents the percentage of replicates which went extinct per treatment for each experiment. A regression line is shown in blue. A GLM using a binomial distribution was run on extinction (presence or absence per replicate) and either PIS or FCR and significance of the interaction is shown in the top left of each panel (see Materials and Methods, see Table S13). Panels (B) and (D) report the influence of drug interaction (PIS and FCR) on cumulative AUC throughout the entire evolution experiment (see Materials and Methods). All treatments included in the combination regimen for which the PIS and FCR scores were available were taken from the data. Each point represents the mean of all replicates in a single treatment for one experiment, with error bars representing the standard error of the mean (n=8-12 replicates per treatment, n = 2 treatments per experiment). A regression line is shown in blue. A linear mixed effects model was performed on the cumulative AUC and drug interaction score for each replicate, and significance of the interaction of tested dependent and explanatory variables is shown in the top left of each panel (see Materials and Methods, see Table S13).

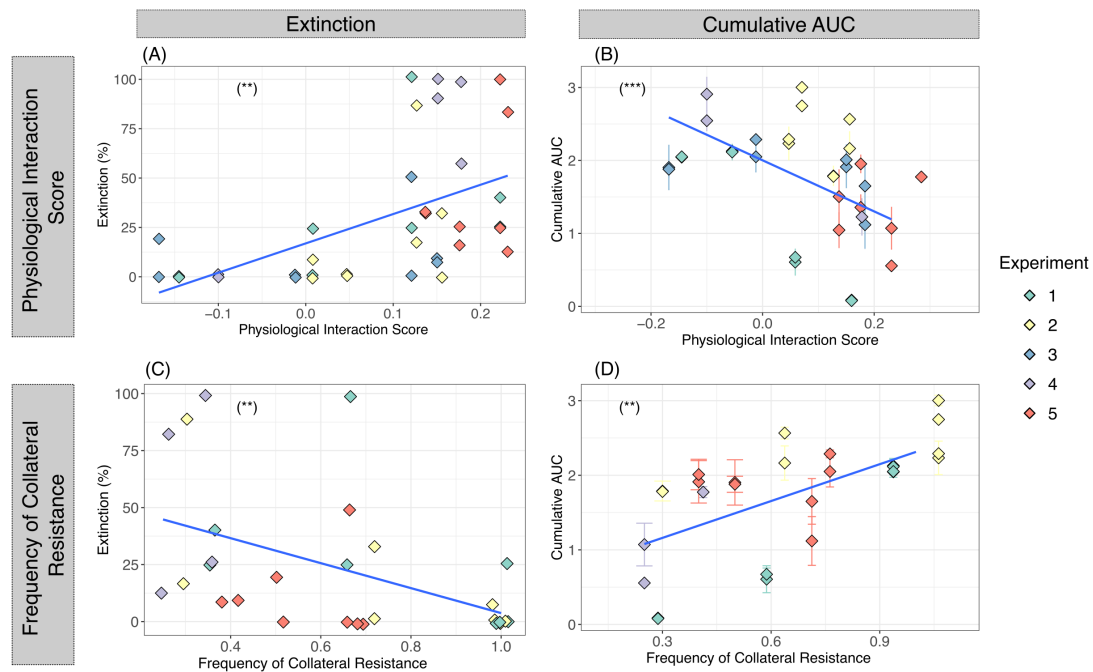


Figure S4 Associations between the applied drug interactions (both physiological and evolutionary interactions) and treatment success (extinction rate and cumulative AUC) when cycling single drugs. Physiological and evolutionary interactions were previously measured for the utilized PA14 ancestor^{198,199} (see Materials and Methods). Panels (A) and (C) show how the physiological interaction score (PIS) and frequency of collateral resistance (FCR) influenced extinction rates. All treatments included in the cycling regimen for which the PIS and FCR scores were available (n=8-12 replicates per treatment, n = 3-8 replicates per experiment, depending on if all interactions were available) were taken from the data. Each point represents the percentage of replicates which went extinct per treatment for each experiment. A regression line is shown in blue. A GLM using a binomial distribution was run on extinction (presence of absence per replicate) and either PIS or FCR, and significance of the interaction is shown in the top left of each panel (see Materials and Methods, see Table S13). Panels (B) and (D) report the influence of drug interaction (PIS and FCR) on cumulative AUC throughout the entire evolution experiment (see Materials and Methods). All treatments included in the cycling regimen for which the PIS and FCR scores were available were taken from the data. Each point represents the mean of all replicates in a single treatment for one experiment, with error bars representing the standard error of the mean (n=8-12 replicates per treatment, n = 3-8 replicates per experiment, depending on if all interactions were available). A regression line is shown in blue. A linear mixed effects model on the cumulative AUC and drug interaction score for each replicate, and significance of the interaction of tested dependent and explanatory variables is shown in the top left of each panel (see Materials and Methods, see Table S13).

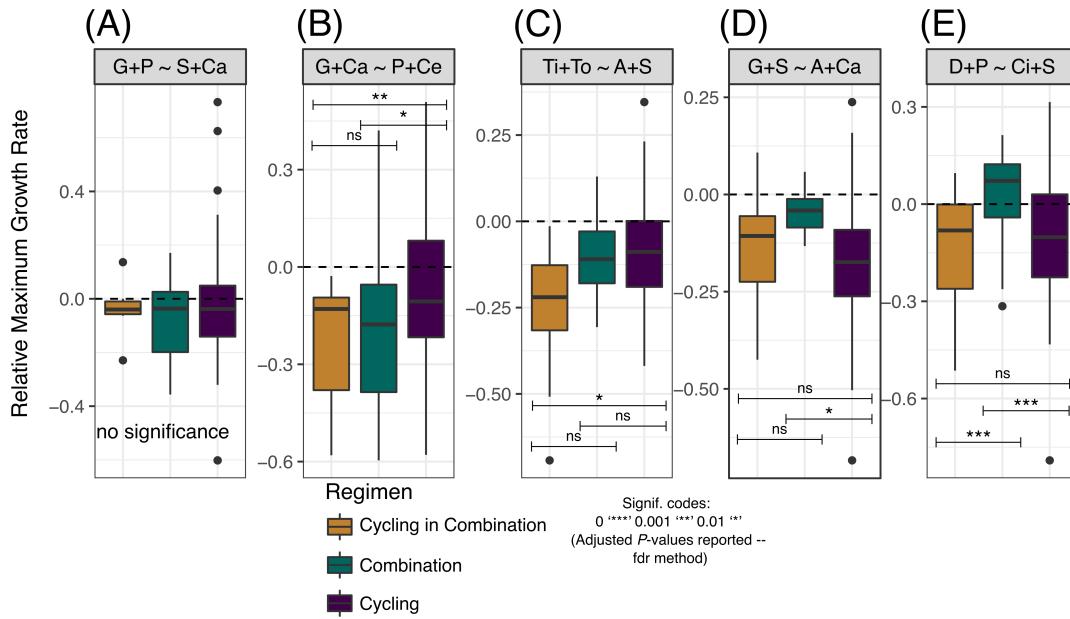


Figure S5 Relative maximum growth rate under antibiotic-free conditions (A-E). Maximum growth rate was calculated using the R package ‘grofit’²⁵⁹, and was made relative to the evolving control, evolving in no-drug medium. A linear mixed model was performed on each evolution experiment separately to test if the differences between the regimens were significant. Treatment was used as the fixed factor, relative maximum yield as the response variable, and row in plate was a random factor. Differences between regimens were tested via a multiple comparisons post hoc test, and were corrected for multiple testing using fdr (see Materials and Methods).

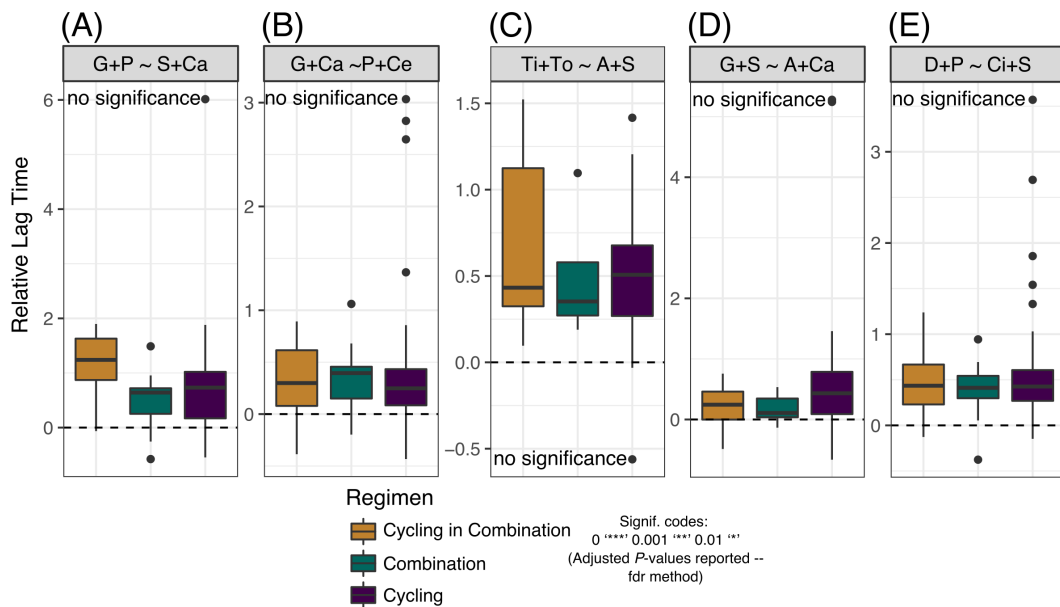


Figure S6 Relative lag time under antibiotic-free conditions (A-E). Relative lag time was measured using the ‘grofit’ package²⁵⁹, and relative measurements were calculated from the evolving control in no drug media. A linear mixed model was performed on each evolution experiment separately to test if the differences between the regimens were significant. Treatment was used as the fixed factor, relative lag time as the response variable, and row in plate was a random factor. Differences between regimens were tested via a multiple comparisons post hoc test, and were corrected for multiple testing using fdr (see Materials and Methods).

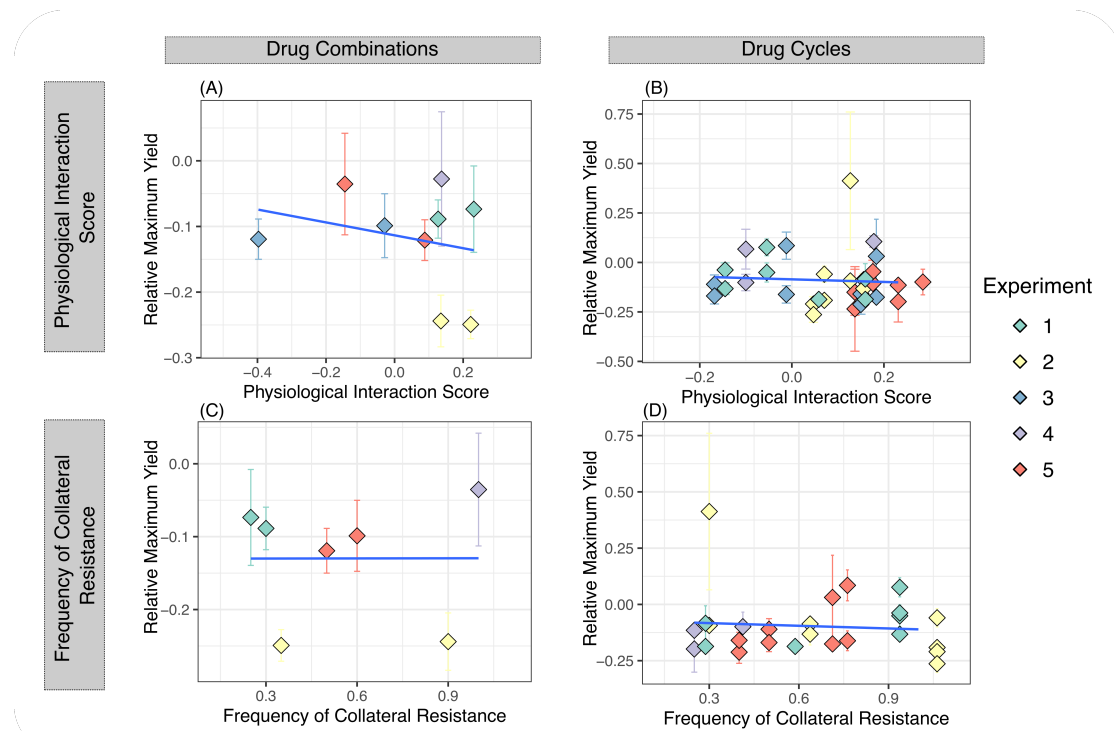


Figure S7 Associations between the applied drug interactions (both physiological and evolutionary interactions) and fitness costs for cycling and combination regimens. Physiological and evolutionary interactions were previously measured for the utilized PA14 ancestor^{198,199} (see Materials and Methods). Panels (A) and (C) show how the physiological interaction score (PIS) and frequency of collateral resistance (FCR) influenced fitness costs (measured by relative maximum yield) for drug combinations. All treatments included in the combination regimen for which the PIS and FCR scores were available (n=8-12 replicates per treatment per experiment) were taken from the data. Each point represents the mean relative maximum yield per treatment for each experiment, with error bars representing standard error of the mean. A regression line is shown in blue. Panels (B) and (D) report the influence of drug interaction (PIS and FCR) on fitness costs for populations exposed to drug cycles. All treatments included in the cycling regimen for which the PIS and FCR scores were available were taken from the data. Each point represents the mean of all replicates in a single treatment for one experiment, with error bars representing the standard error of the mean (n=8-12 replicates per treatment per experiment). A regression line is shown in blue.

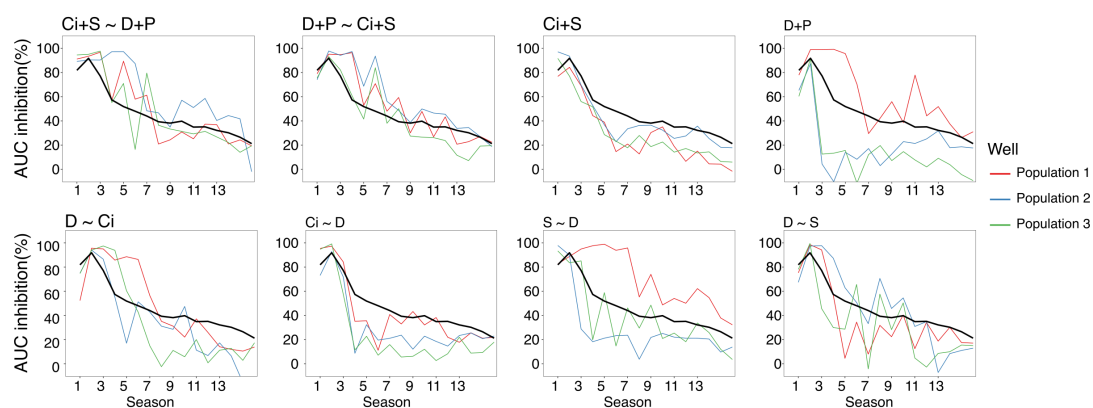


Figure S8 AUCi dynamics of select populations chosen to be further characterized. Three populations (3 replicate wells) from each treatment were chosen from each of the above treatment protocols. The three wells were selected based on the presence or absence of zigzag dynamics in addition to the difference in AUCi from the mean. By attempting to pick wells displaying various adaptive patterns, the aim of subsequent characterization was to distinguish if replicate populations showing the zigzag dynamics had more or less population heterogeneity.

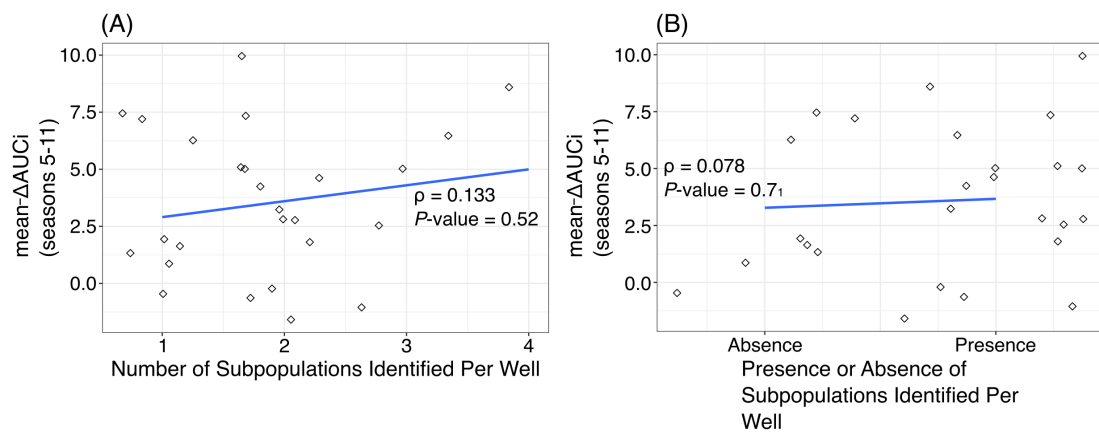


Figure S9 Correlations between subpopulation data and ΔAUC_i . (A) The maximum number of clusters identified per population was extracted from the hierarchical clustering dataset and a Spearman rank's correlation was run against previously measured ΔAUC_i as a proxy for zigzag presence and extent. Regression line is shown in blue, and Spearman rank coefficient ρ and associated P -values are shown in the panel. (B) The presence or absence of bacteria subpopulations was extracted from the hierarchical clustering data set and denoted by absence (0) or presence (1) of two or more subpopulations. A Spearman's rank correlation was subsequently run against ΔAUC_i . Regression line is shown in blue, and Spearman rank coefficient ρ and associated P -values are shown in the panel.

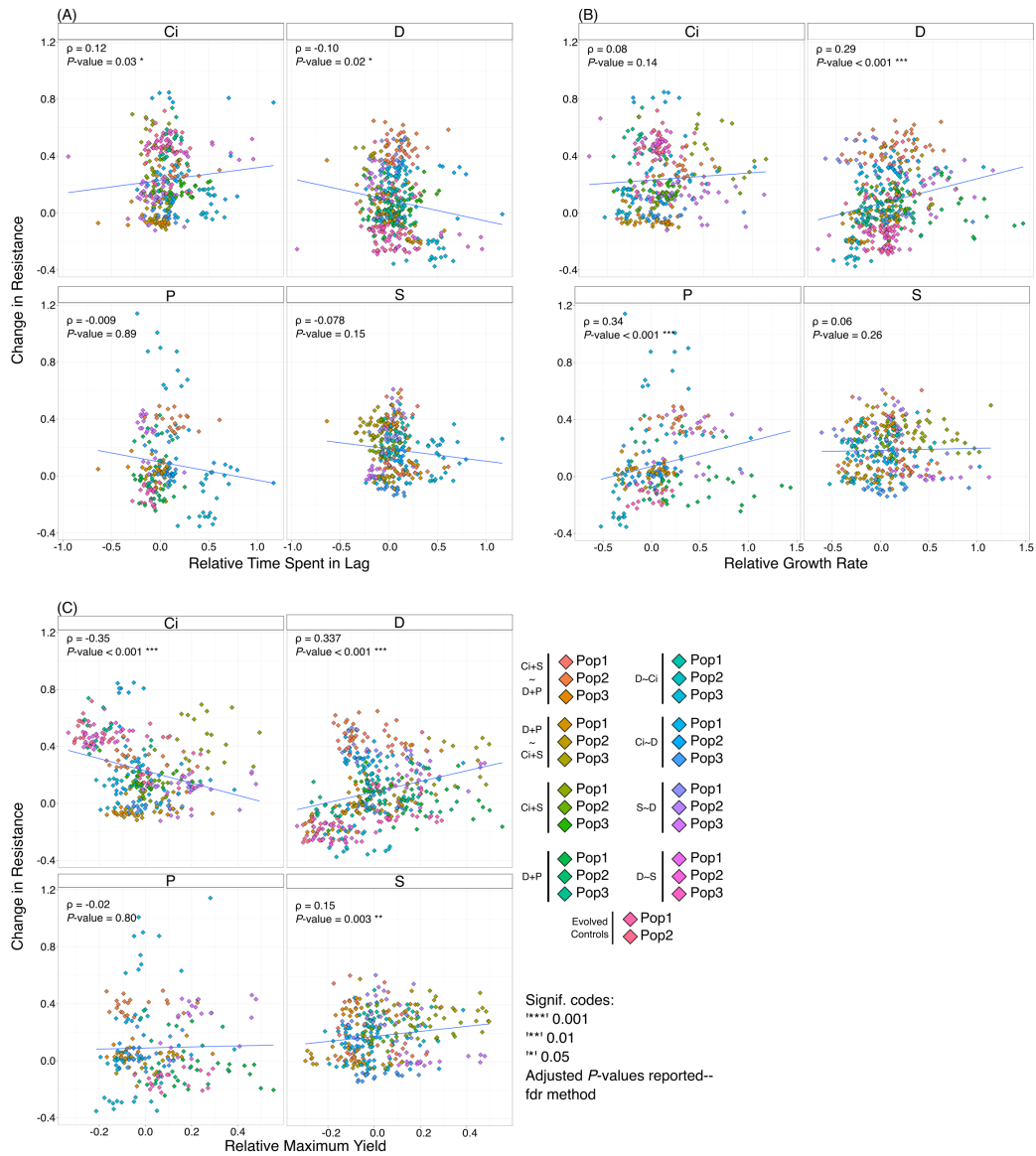


Figure S10 Fitness proxies correlated with change in resistances for characterized evolved isolates. Previously calculated changes in resistance and fitness costs were extracted for each colony in the characterized evolved isolates from season 14 (see Methods). Using a Spearman correlation, relative time spent in lag phase (A), relative growth rate (B), and relative maximum yield (C) were correlated with the change of resistance seen for each antibiotic in the individual colonies relative to the PA14 ancestor strain. Regression lines are shown in blue, and Spearman's correlation coefficient and fdr-corrected P -values are shown in the top left panels.

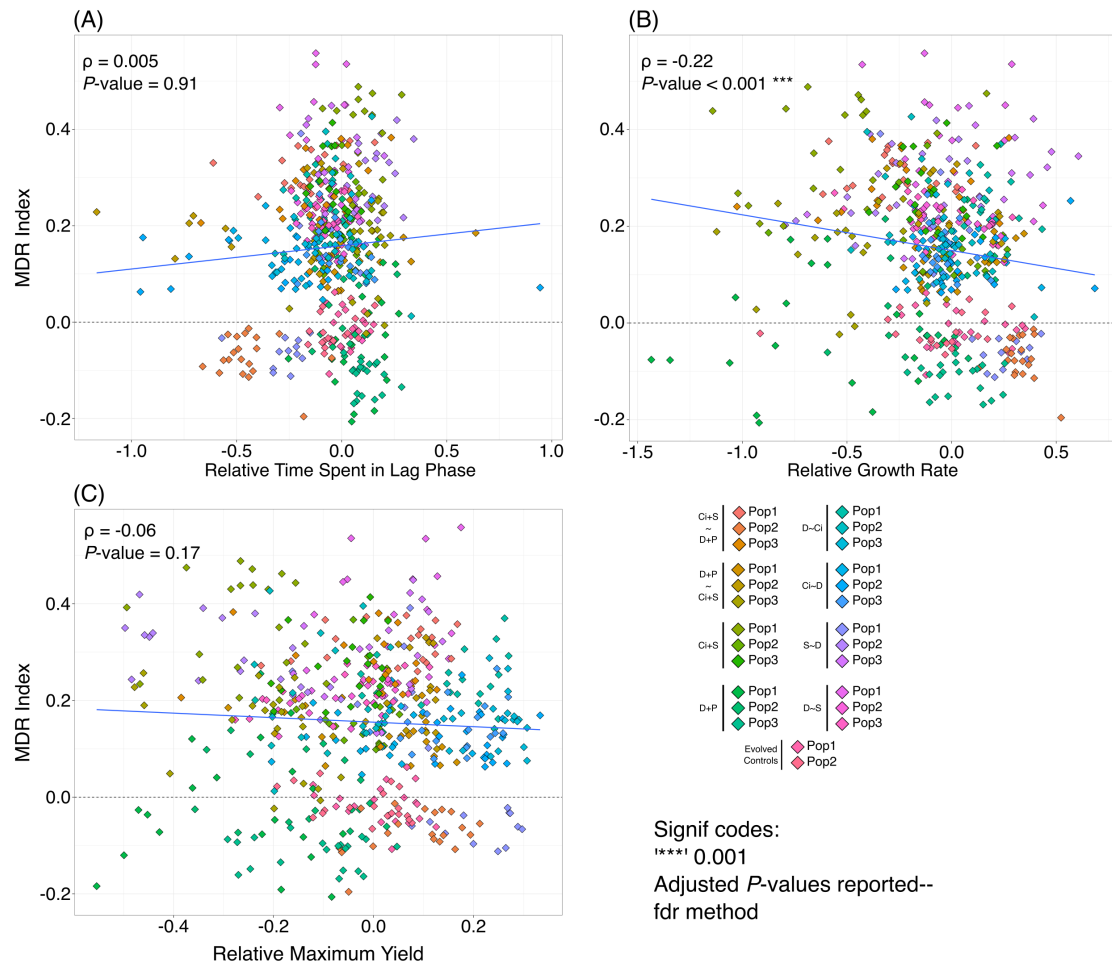


Figure S11 Multidrug resistance indices correlated with fitness costs. Previously calculated MDR indices were extracted for each of the colonies in the characterized evolved material from season 14. A Spearman's correlation was performed to test for a relationship between presence and extent of multidrug resistance and an associated fitness cost. Relative lag time (A), relative growth rate (B), and relative maximum yield (C) were tested against MDR indices for each isolate. Regression lines are shown in blue, and Spearman's correlation coefficient as well as fdr-corrected *P*-values are shown in the top left of each panel.

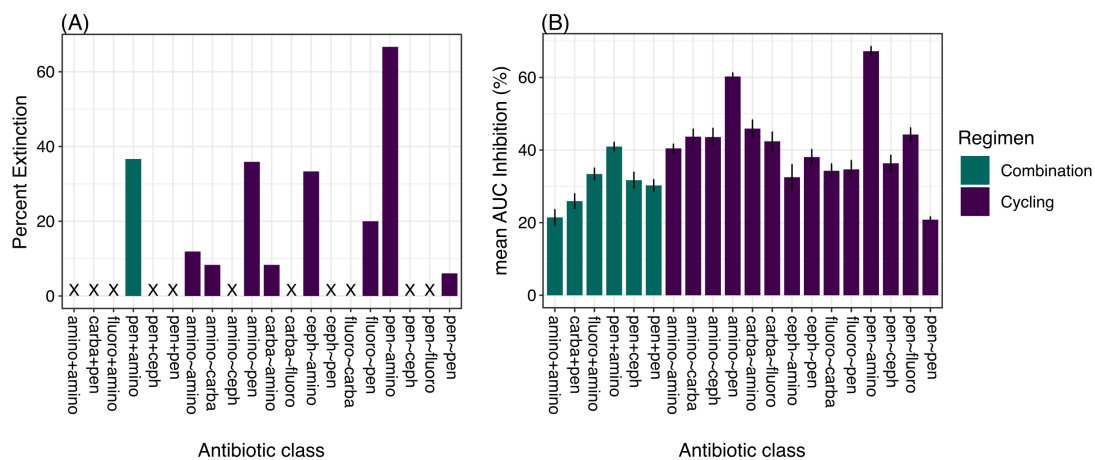


Figure S12 Impact of antibiotic class on extinction rates and growth inhibition. Antibiotic abbreviations are as follows: amino, aminoglycoside; carba, carbapenem; pen, penicillin; fluoro, fluoroquinolone; ceph, cephalosporin. (A) All lineages from every experiment were taken and first established if living or extinct (see Methods). Next, the number of extinctions per antibiotic class (of both utilized antibiotics) were counted and are expressed as a

percentage above. **(B)** AUC inhibitions (AUC_i) were standardized for every experiment based on the relevant evolved controls (see Methods). Lineages from all experiments were extracted and the mean of all Lineages was calculated and expressed as an overall percentage above. Error bars represent standard error of the mean.

Supplementary Tables

Table S1 Treatments selected for subsequent evolved material characterization

Regimen	Corresponding Antibiotics
Cycling in Combination	D+P ~ Ci+S
Cycling in Combination	Ci+S ~ D+P
Combination	D+P
Combination	Ci+S
Cycling	D ~ Ci
Cycling	Ci ~ D
Cycling	D ~ S
Cycling	S ~ D

Table S2 Significant differences in cumulative AUC between drug regimens

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
Cycling in Combination versus Combination	0.344	0.137	< 0.001 ***	< 0.001***	< 0.001***
Cycling in Combination versus Cycling	0.112	0.017*	0.248	0.821	0.016*
Cycling versus Combination	0.130	0.396	0.112	< 0.001***	< 0.001***

The table shows *P*-values adjusted by *fdr* (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

The models consider seasons as an interacting factor, replicate populations as a random factor, cumulative AUC as the response variable, and treatment as the fixed factor. From this model, regimen values were compared using the Tukey's honest significance difference test, shown in the above table.

Table S3 Significant differences in extinction rates between drug regimens

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
Cycling in Combination versus Combination	0.0055**	0.369	0.788*	NA	NA
Cycling in Combination versus Cycling	0.0065**	0.0495*	0.788	0.35	0.461
Cycling versus Combination	0.2072	0.369	0.927	NA	NA

The table shows *P*-values adjusted by fdr (**P* < 0.05, ***P* < 0.01, ****P* < 0.001). The models consider extinction as the response variable and regimen as the explanatory variable. The above *P*-values are the result from a Tukey's honest significance difference test following the GLM.

Table S4 Significant differences in relative maximum yields between drug regimens

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
Cycling in Combination versus Combination	0.363	0.649	0.037*	0.559	0.854
Cycling in Combination versus Cycling	0.349	0.531	0.0084**	0.839	0.854
Cycling versus Combination	0.987	0.389	0.684	0.559	1.000

The table shows *P*-values adjusted by fdr (**P* < 0.05, ***P* < 0.01, ****P* < 0.001). The models consider relative maximum yield as the response variable, treatment as the fixed factor, and position in plate as random factor. From this model, regimen values were compared using the Tukey's honest significance difference test, shown in the above table.

Table S5 Significant differences in relative growth rates between drug regimens

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
Cycling in Combination versus Combination	0.642	0.393	0.0597	0.203	< 0.001***
Cycling in Combination versus Cycling	0.945	0.005**	0.0120*	0.460	0.231
Cycling versus Combination	0.567	0.039*	0.779	0.021*	< 0.001***

The table shows *P*-values adjusted by fdr (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

The models consider relative growth rate as the response variable, treatment as the fixed factor, and position in plate as random factor. From this model, regimen values were compared using the Tukey's honest significance difference test, shown in the above table.

Table S6 Significant differences in relative times spent in lag phase between drug regimens

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
Cycling in Combination versus Combination	0.152	0.964	0.680	0.966	0.468
Cycling in Combination versus Cycling	0.581	0.964	0.273	0.119	0.776
Cycling versus Combination	0.124	0.964	0.680	0.119	0.282

The table shows *P*-values adjusted by fdr (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

The models consider relative lag time as the response variable, treatment as the fixed factor, and position in plate as random factor. From this model, regimen values were compared using the Tukey's honest significance difference test, shown in the above table.

Table S7 Significance of correlation between measured phenotypes in all evolution experiments performed by a Kendall's rank correlation test.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
Cumulative AUC versus Extinction	<i>P</i> -value = 0.0774 tau = -0.4076	<i>P</i> -value = 0.0455* tau = -0.4606	<i>P</i> -value = 0.0034** tau = -0.8693	<i>P</i> -value = 0.0440* tau = -0.5185	<i>P</i> -value = 0.1017 tau = -0.3909
Cumulative AUC versus Relative Maximum Yield	<i>P</i> -value = 0.02878* tau = 0.5138	<i>P</i> -value = 1 tau = 0	<i>P</i> -value = 0.3988 tau = 0.2857	<i>P</i> -value = 0.0286* tau = 0.5556	<i>P</i> -value = 0.1646 tau = 0.3455
Extinction versus Relative Maximum Yield	<i>P</i> -value = 0.2559 tau = -0.2809	<i>P</i> -value = 0.4751 tau = 0.1645	<i>P</i> -value = 0.2518 tau = -0.3402	<i>P</i> -value = 0.36 tau = -0.2357	<i>P</i> -value = 0.3184 tau = 0.2497

The table shows *P*-values adjusted by fdr (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

Table S8 Significant differences between mean IC₅₀ values measured per antibiotic at 12 and 24-hour season lengths performed by a Wilcoxon rank sum test.

Antibiotic	Test statistic (W)	<i>P</i> -value
Ca	W = 1.5	0.063
G	W = 1.5	0.010 **
P	W = 14	0.567
S	W = 8	0.127

Tests for differences were performed separately for each antibiotic (***P* < 0.01)

Table S9 Significant differences in MDR between regimens

Regimens tested	<i>P</i> -value
Cycling in Combination versus Combination	0.997 'ns'
Cycling in Combination versus Cycling	2.32x10 ⁻⁹ ****
Cycling versus Combination	2.32x10 ⁻⁹ ****

The table shows *P*-values adjusted by fdr ('ns' not significant, ****P* < 0.001).

A mixed linear model assessed differences in multi-drug resistance indices per regimen, in which MDR index was the response variable, regimen was the fixed factor, and transfer was a random factor. HSD was used to evaluate significance of differences.

Table S10 Significant differences between changes in resistances between seasons 7 and 14

	D	P	Ci	S
Season 7 vs Season 14	<i>P</i> -value = 0.59 Z = 0.59	<i>P</i> -value = 0.555 Z = 0.59	<i>P</i> -value = 0.000159 **** Z = 3.776	<i>P</i> -value = 0.214 Z = -1.243

The table shows *P*-values adjusted by *fd*r ('ns' not significant, ****P* < 0.001). Significant differences between seasons were investigated by performing a mixed linear model where change in resistance per antibiotic was the response variable, season was the fixed factor, and regimen was a random factor. HSD was used to evaluate significance of differences and *P*-values were corrected by *fd*r.

Table S11 Extinction percentages reported for individual treatments.

Drug Order	Extinction rate (%)	Antibiotic 1 Class	Antibiotic 2 Class	Collateral profile
1 G~S	0	Aminoglycoside	Aminoglycoside	Collateral resistance
1 S~G	0	Aminoglycoside	Aminoglycoside	Collateral resistance
1 G~Ca	40	Aminoglycoside	Penicillin	Collateral resistance
1 Ca~G	25	Penicillin	Aminoglycoside	NS
1 P~S	100	Penicillin	Aminoglycoside	NS
1 S~P	25	Aminoglycoside	Penicillin	Collateral resistance
1 P~Ca	0	Penicillin	Penicillin	Collateral resistance
1 Ca~P	25	Penicillin	Penicillin	Collateral resistance
2 G~P	16.7	Aminoglycoside	Penicillin	NS
2 P~G	87.5	Penicillin	Aminoglycoside	NS
2 G~Ce	0	Aminoglycoside	Penicillin	Collateral resistance
2 Ce~G	33.3	Penicillin	Aminoglycoside	NS
2 Ca~P	8.33	Penicillin	Penicillin	Collateral resistance
2 P~Ca	0	Penicillin	Penicillin	Collateral resistance
2 Ca~Ce	0	Penicillin	Cephalosporin	Collateral resistance
2 Ce~Ca	0	Cephalosporin	Penicillin	Collateral resistance
3 Ti~A	0	Penicillin	Penicillin	NA
3 A~Ti	0	Penicillin	Penicillin	NA
3 Ti~S	100	Penicillin	Aminoglycoside	NA
3 S~Ti	58.3	Aminoglycoside	Penicillin	NA
3 To~A	100	Aminoglycoside	Penicillin	NA
3 A~To	91.7	Penicillin	Aminoglycoside	NA
3 To~S	33.3	Aminoglycoside	Aminoglycoside	NA
3 S~To	8.3	Aminoglycoside	Aminoglycoside	NA
4 G~A	25	Aminoglycoside	Penicillin	NA
4 A~G	16.7	Penicillin	Aminoglycoside	NA
4 G~Ca	25	Aminoglycoside	Penicillin	Collateral resistance
4 Ca~G	100	Penicillin	Aminoglycoside	NS
4 S~A	33.3	Aminoglycoside	Penicillin	NA
4 A~S	33.3	Penicillin	Aminoglycoside	NA
4 S~Ca	12.5	Aminoglycoside	Penicillin	NS
4 Ca~S	83.3	Penicillin	Aminoglycoside	NS
5 D~Ci	0	Carbapenem	Fluoroquinolone	NS
5 Ci~D	0	Fluoroquinolone	Carbapenem	Collateral resistance
5 D~S	8.3	Carbapenem	Aminoglycoside	Collateral sensitivity
5 S~D	8.3	Aminoglycoside	Carbapenem	NS
5 P~Ci	0	Penicillin	Fluoroquinolone	Collateral resistance
5 Ci~P	20	Fluoroquinolone	Penicillin	Collateral sensitivity
5 P~S	50	Penicillin	Aminoglycoside	NS
5 S~P	0	Penicillin	Penicillin	Collateral resistance

Highlighted rows represent differences in extinction rate of at least 20% for antibiotics used in reverse order. NS represents no significant collateral profile measured during analyses¹⁹⁹. NA represents pairs that were not measured during analyses and to which collateral profiles are not known. Drug abbreviations are shown in Table 2.

Table S12 Evolutionary interactions of drug combinations.

Experiment	Component Drugs	Collateral profile
1	G+P	Partial collateral sensitivity
1	S+Ca	Collateral resistance
2	G+Ca	Partial collateral sensitivity
2	P+Ce	Collateral resistance
3	Ti+To	NA
3	A+S	NA
4	G+S	Collateral resistance
4	A+Ca	NA
5	D+P	Partial collateral sensitivity
5	Ci+S	Partial collateral resistance

Evolutionary interactions of drug combinations were measured in previous work¹⁹⁹, and antibiotic abbreviations are shown in Table 2.

Table S13 Influences of drug interactions on extinction rate and cumulative AUC.

Tested regimen	Explanatory variable	Response Variable	Formula	Fixed effects table				
Combination	PIS	Extinction	Extinction ~ PIS + (1 Experiment) + (1 Treatment)	(Intercept)	Estimate	Std. Error	z value	Pr(> z)
				PIS	-14.08 63.91	8.13 41.41	-1.732 1.543	0.0833 (.) 0.1227 (ns)
Combination	FCR	Extinction	Extinction ~ FCR + (1 Experiment) + (1 Treatment)	(Intercept)	Estimate	Std. Error	z value	Pr(> z)
				FCR	3.828 -17.960	4.267 13.536	0.897 -1.327	0.370 (ns) 0.185 (ns)
Combination	PIS	Cumulative AUC	Cumulative AUC ~ PIS + (1 Experiment) + (1 Treatment)	(Intercept)	Estimate	Std. Error	t value	Pr(>Chisq)
				PIS	2.3807 -1.2549	0.1240 0.5934	19.197 -2.115	0.034(*)
Combination	FCR	Cumulative AUC	Cumulative AUC ~ FCR + (1 Experiment)	(Intercept)	Estimate	Std. Error	t value	Pr(>Chisq)
				FCR	1.6440 1.1968	0.1967 0.2606	8.360 4.593	4.38e-06(***)
Cycling	PIS	Extinction	Extinction ~ PIS + (1 Experiment) + (1 Treatment)	(Intercept)	Estimate	Std. Error	z value	Pr(> z)
				PIS	-3.0275 14.1549	0.8737 4.5217	-3.465 3.130	0.00053 (***) 0.00175 (**)
Cycling	FCR	Extinction	Extinction ~ FCR + (1 Experiment) + (1 Treatment)	(Intercept)	Estimate	Std. Error	z value	Pr(> z)
				FCR	1.363 -5.881	1.374 2.173	0.992 -2.706	0.32130 (ns) 0.00681 (**)
Cycling	PIS	Cumulative AUC	Cumulative AUC ~ PIS + (1 Experiment) + (1 Treatment)	(Intercept)	Estimate	Std. Error	t value	Pr(>Chisq)
				PIS	2.0451 -3.8921	0.2079 0.7515	9.838 -5.179	2.658e-09(**)
Cycling	FCR	Cumulative AUC	Cumulative AUC ~ FCR + (1 Experiment) + (1 Treatment)	(Intercept)	Estimate	Std. Error	t value	Pr(>Chisq)
				FCR	0.4989 2.0917	0.3414 0.3515	1.461 5.951	2.23e-08(**)

A GLM with a binomial distribution was performed to measure the influence of drug interactions (both physiological interaction score (PIS) and frequency of collateral resistance (FCR)) on cumulative AUC and extinction rates. A linear mixed model modeled an association between drug interactions (both PIS and FCR) on cumulative AUC and extinction rate, *P*-values on the model were obtained by running a Wald test on the model using the Anova function in the ‘car’ package²⁶⁰.

Table S14 Influences of drug interactions on fitness costs.

Tested regimen	Explanatory variable	Response Variable	Model 1	ANOVA results between null and Model 1						
				Null	Df	AIC	BIC	logLike	deviance	Pr(>Chisq)
Combination	Physiological interaction score	Relative maximum yield	Relative maximum yield ~ PIS + (1 Experiment)	Null	3	-82.5	-75.6	44.2	-88.5	0.79 (ns)
				Model 1	4	-80.6	-71.4	44.3	-88.6	
Combination	FCR	Relative maximum yield	Relative maximum yield ~ FCR + (1 Experiment)	Null	3	-69.1	-62.7	37.5	-75.1	0.86 (ns)
				Model 1	4	-67.1	-58.6	37.6	-75.1	
Cycling	Physiological interaction score	Relative maximum yield	Relative maximum yield ~ PIS + (1 Treatment)	Null	3	86.2	96.8	-40.1	80.2	0.88 (ns)
				Model 1	4	88.2	102.2	-40.1	80.2	
Cycling	FCR	Relative maximum yield	Relative maximum yield ~ FCR + (1 Treatment)	Null	3	90.0	99.97	-41.99	84.0	0.50 (ns)
				Model 1	4	91.5	104.9	-41.77	83.5	

A linear mixed model was compared to a null model, and significance of differences between the tested model and null model were assessed using an ANOVA.

Table S15 Significant differences between Shannon diversity index of regimens

Regimens tested	<i>P</i> -value
Cycling in Combination versus Combination	0.25 **
Cycling in Combination versus Cycling	0.02 **
Cycling versus Combination	0.07 'ns'

The table shows *P*-values adjusted by fdr ('ns' not significant, **P* < 0.05, .*P* < 0.1).

A linear model was performed to assess significant differences between population heterogeneity with regimens. Shannon diversity index was the response variable, regimen was the fixed factor, and transfer was considered a random factor. Significance of differences was evaluated via HSD.

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