

Adaptation of Bacteria to Temporally Changing Antibiotic Environments

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Declaration

I, **Roderich Römheld**, declare that:

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

Specific aspects of my thesis were supported by colleagues; their contribution is specified in detail in the following section "Author's contributions";

The thesis has not already been submitted neither partially nor wholly as part of a doctoral degree to another examining body.

The thesis has not been published, but indicated parts of the thesis have been submitted for publishing;

The thesis has been prepared subject to the Rules of Good Scientific Practice of the German Research Foundation (DFG).

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Author's contributions

This thesis consists of four chapters; each represented by a manuscript. Roderich Römhild developed original ideas and wrote the manuscripts with major contributions.

Chapter 1: Evolutionary ecology meets the antibiotic crisis: Can we control evolution? Roderich Römhild and Hinrich Schulenburg.

RR and HS jointly conceptualized the idea, reviewed the relevant literature, and wrote the manuscript.

Chapter 2: Adaptive paths to escape collateral sensitivity cycling. Roderich Römhild*, Camilo Barbosa*, Philip Rosenstiel, and Hinrich Schulenburg.

RR and CB contributed equally to this work (*) by conceptualizing the idea for the project, performing the experiments, analyzing and interpreting the data, and writing the manuscript.

PR provided material and sequencing services, and discussed the data.

HS conceptualized the idea for the project, supervised the project, and discussed and wrote the manuscript.

Chapter 3: Negative hysteresis improves antibiotic cycling efficacy. Roderich Römhild, Chaitanya S. Gokhale, Christopher Blake, Philip Rosenstiel, Arne Traulsen, Dan I. Andersson, and Hinrich Schulenburg.

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PR provided material and sequencing services, and discussed the data.

RR, DIA and HS conceptualized the idea for the project, and wrote the manuscript.

All authors approved the manuscript.

Chapter 4: Sequential treatment with three β -lactams in *Pseudomonas aeruginosa* and the evolution of resistance. Roderich Römhild and Hinrich Schulenburg.

RR performed the experiments and analyzed the data.

RR and HS conceptualized the idea for the project, and wrote the manuscript.

As supervisor I confirm the above stated contributions

Signature: _____

“Biologists are observing year by year and sometimes even day by day or hour by hour details of life’s unrolling and opening, right now.”

Jonathan Weiner

The Beak of the Finch, 1994

“... the more you look the more you see.”

Peter Grant



Contents

Declaration	3
Author's contributions	4
Contents	7
Summary	8
Deutsche Zusammenfassung	9
Introduction	10
Chapter 1	31
Evolutionary ecology meets the antibiotic crisis: Can we control evolution?	
Chapter 2	48
Adaptive paths to escape collateral sensitivity cycling.	
Chapter 3	69
Negative hysteresis improves antibiotic cycling efficacy.	
Chapter 4	114
Sequential treatment with three β -lactams in <i>Pseudomonas aeruginosa</i> and the evolution of resistance.	
General discussion	120
List of abbreviations	130
Special devices, materials and chemicals	131
Acknowledgements	133
Curriculum vitae	134

Summary

The variability of the environment is a challenge for the flexibility of organisms. Temporal variation generates interesting optimization conflicts for evolution, which I investigated in this doctoral thesis for the example of *Pseudomonas aeruginosa* and the sequential treatment with antibiotics. This bacterium has remarkable metabolic and genetic versatility. *P. aeruginosa* expresses a range of efflux pumps for cell detoxification. Increasingly, this characteristic is transforming into a medical threat because it can convey antibiotic resistance. The spread of antibiotic resistance is a growing global challenge. The investigations of this thesis, may contribute to the design of new treatment strategies that inhibit the emergence of resistance. To achieve this aim, we integrated three principles from evolutionary ecology into drug treatments and tested them for their efficacy; we tested genetic conflicts, physiological conflicts, and environmental stochasticity for their ability to delay resistance evolution.

The emergence of resistance can be countered with sequential treatments. Resistance mutations frequently cause hypersensitivity to other antibiotics. The targeted change of antibiotics may thus maintain treatment efficacy in spite of bacterial adaptation. I measured the evolutionary stability of these genetic conflicts, and found that their stability depended on treatment order.

The physiological response to antibiotic stress can temporarily increase the sensitivity against other antibiotics. The integration of such *antibiotic hysteresis* into sequential treatments could inhibit resistance evolution. Selection by hysteresis shifted adaptive priority towards physiological response optimization. My investigations indicate a new treatment strategy that is potentially promising, because it increases the immediate bactericidal effect and prolongs effective treatment by delaying resistance emergence.

Unpredictable environmental variation can complicate evolutionary adaptation by limiting the spectrum of potential adaptive strategies. A decelerating effect was not generally observed in my experiments, but the strongest deceleration of adaptive response occurred in sequential treatments with irregular order of antibiotics. A mathematical model was developed based on these results. The model explained the observed variation between different environmental sequences and accurately predicted the rate of evolutionary adaptive response.

Altogether my experiments emphasize the importance of cellular physiological balance for the evolution of bacteria. My findings may contribute to the development of novel treatment concepts that inhibit the emergence of antibiotic resistance.

< **Picture credit opening page:** Christian Urban, CAU Kiel, 2015

Deutsche Zusammenfassung

Die Variabilität der Umwelt ist eine große Herausforderung für die Flexibilität der Lebewesen. Über lange Zeiträume entstehen interessante Optimierungskonflikte für die Evolution. Diese habe ich im Rahmen dieser Doktorarbeit beispielhaft am Bakterium *Pseudomonas aeruginosa* und mit sequentieller Antibiotikabehandlung untersucht. Die Besonderheit dieses Bakteriums ist seine metabolische und genetische Vielseitigkeit. *P. aeruginosa* besitzt zahlreiche Pumpsysteme zur Entgiftung der Zelle. Zunehmend wird diese Eigenschaft zu einer medizinischen Bedrohung, da durch sie Antibiotikaresistenz vermittelt werden kann. Die Verbreitung von Antibiotikaresistenzen ist eine dringliche globale Herausforderung. Die Untersuchungen dieser Dissertation tragen dazu bei, neue Behandlungsmethoden zu entwickeln, mit denen die Entstehung weiterer Resistenzen gehemmt werden kann. Hierzu wurden Prinzipien der Evolutionsökologie in Antibiotikabehandlungen integriert und auf ihre Wirksamkeit erprobt: Genetische Konflikte, physiologische Konflikte und Stochastizität.

Der Entstehung von Antibiotikaresistenz kann durch sequentielle Behandlung begegnet werden. Viele Resistenzmutationen bewirken Hypersensitivität gegenüber anderen Antibiotika. Auf Grund dieser Tatsache kann durch einen gezielten Wechsel, der Antibiotika, die Wirksamkeit der Behandlung garantiert werden. Im Rahmen meiner Untersuchungen habe ich die evolutionäre Stabilität dieser genetischen Konflikte gemessen und dabei festgestellt, dass eine Abhängigkeit zur Behandlungsreihenfolge besteht.

Die physiologische Einstellung auf Antibiotikastress kann die Empfindlichkeit gegenüber einem anderen Antibiotikum zeitweise erhöhen. Durch den Einbau solcher physiologischen Konflikte in Behandlungsprotokolle, das heißt durch die Berücksichtigung von *Antibiotikahysterese*, ließ sich die Resistenzentstehung hemmen, da sich die Priorität der evolutionären Anpassung zugunsten physiologischer Optimierung verschob. Mit diesen Untersuchungen habe ich einen neuen Behandlungsansatz aufgezeigt, der den unmittelbaren Behandlungserfolg steigern und die langfristige Wirksamkeit gewährleisten könnte.

Unvorhersehbare Umweltvariationen erschweren die evolutionäre Anpassung, da diese das Anpassungsspektrum einschränken. Eine Hemmwirkung von Umweltstochastizität auf Resistenzentwicklung lag in meinen Untersuchungen nicht im Allgemeinen vor, aber die wirksamsten Behandlungssequenzen hatten unregelmäßige Abfolge. Darauf aufbauend wurde ein mathematisches Modell entwickelt, mit dem die Wirksamkeit verschiedenster Behandlungsabfolgen erklärt und vorhergesagt werden konnte.

Insgesamt bestätigen meine Experimente die Wichtigkeit des physiologischen Gleichgewichts der Zelle für die Evolution von Bakterien. Auf Grundlage meiner Untersuchungen können neue Behandlungskonzepte abgeleitet werden, die die Entstehung von Antibiotikaresistenz hemmen.

Introduction

Natural environments are highly dynamic; they are ever-changing due to seasonal and circadian rhythms, day-to-day fluctuations and micro-scale gradients. The resulting environmental contrasts affect the fitness of organisms and thereby exert selection. Frequent disturbances interfere with offspring production, either directly through temporary resource availability, or indirectly by increasing resource demand through stress. Less frequent disturbances that span several generations, occasionally invert the direction of selection, potentially complicating adaptation. Thus, species face the challenge of keeping tune with their dynamic surroundings, for which some have evolved fascinatingly complex mechanisms. This dissertation presents the results of my investigations on how different aspects of temporal variation affect the evolutionary emergence of novel traits in the bacterium *Pseudomonas aeruginosa*. I was particularly interested in the importance of temporal regularity, and phenotypic memory for the rate and scope of adaptation, which I investigated with evolution experiments and artificial selection by sequential exposure to antibiotics. The approach was complemented by physiological characterization, genetic analyses, statistics, and mathematical modeling.

In this first chapter, I provide background on fluctuating environments. I discuss the physiological responses of bacteria to sudden environmental contrasts and outline how recurrent change can be a potent selective force that drives, yet also complicates, evolutionary adaptation. For feasibility, the discussion is restricted to prokaryotes, especially bacteria. Nevertheless, many aspects are relevant for eukaryotes as well. I conclude this chapter by introducing the model system and indicating how my work may contribute to the design of evolution-robust antibiotic therapy. The potential applications of my findings are discussed in more detail in a separate chapter, at the end of the dissertation.

Temporal variation

Most microbial environments fluctuate on a daily basis. Because the fluctuations are often caused by the movements of the earth or moon, it is difficult to find a microbial habitat devoid of temporal structure. Perhaps the closest approximations are permafrost soils. The majority of microbial habitats are, however, characterized by frequent fluctuations. For example, tidal rhythms shift shoreline ecosystems between aquatic and terrestrial states, twice daily. Bacteria in mangrove sediments are thus exposed to rapid drops and increases of salinity (Barr *et al.*, 2010). Further temporal contrasts arise from spatial variation and micro-scale gradients, which bacteria encounter during movement and translocation. Rapid transitions between environmental contrasts, thus, occur for gut symbionts that passage through a specific nutrient sequence along our digestive tract (Savageau, 1998), and for pathogens that switch between external and within-host environments (Schild *et al.*, 2007). In summary, temporal variation is a pervasive feature of bacterial habitats, and a systematic understanding of the ensuing ecological challenges is key to the understanding of bacterial evolution.

Table 1. Fine-scale temporal structure of natural environments of bacteria.

Environment	Fluctuating parameter	Trigger	Scale[†]	Predictability[‡]	Reference
Rainforest soil	oxygen	rain	between days	U	Silver <i>et al.</i> , 1999
Productive lake	pH	rain	between days and diurnal	U	Maberly, 1996
Ocean floor sediment	marine snow	light	between days and diurnal	U, structure within days	Lampitt <i>et al.</i> , 1993
Waste water	antibiotics	human activity	diurnal	mostly P	Coutu <i>et al.</i> , 2013
Mangrove sediment	salinity	tides	tidal	P	Barr <i>et al.</i> , 2010
Wetland plant rhizosphere	redox	light	diurnal	P	Nikolausz <i>et al.</i> , 2008
Seagrass rhizosphere	redox: iron (II), sulfide	light	diurnal	P	Pagès <i>et al.</i> , 2012
Salt marsh	redox: iron (II), sulfide	light	diurnal	P	Luther & Church, 1988
Hypersaline microbial mat	redox: iron (II), sulfide	light	diurnal	P	Pages <i>et al.</i> , 2014
Hypersaline lake cyanobacterial mat	oxygen, redox, H ₂ S	light	diurnal	P	Jørgensen <i>et al.</i> , 1979
Hot spring microbial mat	oxygen	light	diurnal	P	Steunou <i>et al.</i> , 2008
Hyperventilating shallow estuary	oxygen	light	diurnal	P	Beck & Bruland, 2000
Streambed during algal bloom	acetate	light	diurnal	P	Kaplan & Bott, 1989
Cobble-bed river close to mine	dissolved metal ions, nitrate	microbial respiration	diurnal	P	Brick & Moore, 1996
Polluted high altitude lake	mercury	unclear	diurnal	P	Alanoca <i>et al.</i> , 2016
Host colonization	oxygen, temperature	spatial variation	sudden	P	Schild <i>et al.</i> , 2007; Tagkopoulos <i>et al.</i> , 2008
Mammalian gut	nutrients (sugars)	digestion	hours	P	Savageau, 1998

[†] Seasonal variation excluded. [‡] Temporal regularity of fluctuations: P, predictable; U, unpredictable.

An important parameter governing adaptation to fluctuating parameters may be their predictability (Kashtan, Noor, & Alon, 2007; Mitchell & Pilpel, 2011; Botero *et al.*, 2015), because it provides opportunity for the evolution of pre-adaptation. Predictable periodic fluctuations are often triggered by oscillating external factors, most notably light. The light-dependent metabolic activity of plants, animals, and bacteria generates diurnal structure in environments ranging from the deep-sea to rainforest soil (Table 1). Diurnal variation can occur in any abiotic parameter relevant to the ecology of organisms, including resource supply, toxicity, pH, and redox levels (Table 1). Some diurnal variations are caused by the activity patterns of humans; drug concentrations in waste water have a morning peak (and somewhat smaller evening peak) corresponding to the times of typical toilet use (Coutu *et al.*, 2013). The progression of many diurnal fluctuations can be regarded as a fixed sequence of events. Any single event can potentially serve as a cue for organisms to prepare for following events. Fixed sequential orders are indeed exploited by gut bacteria. Wildtype *Escherichia coli* show lactose-triggered upregulation of maltose genes – in anticipation of maltose rich conditions encountered three hours later (Savageau, 1998; Mitchell *et al.*, 2009). Similarly, *E. coli* prepare for anoxia following rapid temperature increases (Tagkopoulos, Liu, & Tavazoie, 2008), a predictive behavior that reflects their natural coincidence when entering the gut. Anticipatory gene regulation is common in pathogenic bacteria (Brunke & Hube, 2014) yet the costs associated to preparation, restrict its evolution to highly predictable environments (Mitchell & Pilpel, 2011).

The timing of other environmental contrasts is stochastic and thereby devoid of reliable cues. Stochastic fluctuations can be a consequence of unpredictable precipitation (Table 1), which, for example, causes fluctuations of soil oxygen levels by several orders of magnitude (Silver, Lugo, & Keller, 1999). In other cases the occurrence of fluctuations is unpredictable, but their progression is structured. There is unpredictable day-to-day variation in the amount of marine snow in the deep sea, yet morning supply is larger than evening supply (Lampitt, Hillier, & Challenor, 1993). Although unpredictable environmental variation is likely important in the natural ecology of bacteria, its effect on local adaptation is unclear.

Physiological response

Environmental contrasts require specific changes in cellular physiology. Bacteria cannot express their whole genome simultaneously, because many functions are biochemically incompatible, and gene expression is associated with fitness costs. Instead, bacteria selectively express parts of their functional repertoire in response to the current ecological needs. Upon encountering sudden change in environment, they typically reduce growth and enter a temporary lag phase during which there is no increase in cell number (Monod, 1949). The duration of lag phase, i.e. the time for the first generation in the new environment, depends on the disparity of the environmental contrast. For example, growth lagged for 2h after a switch from glucose-depleted media to fresh glucose media, and 8h when cells were switched to arabinose instead (Madar *et al.*, 2013). The seeming inertia is deceiving, as the lag phase is a first and specific step of bacterial response. A switch of carbon sources in the example (glucose -> arabinose) was followed by an immediate exponential increase in the expression of arabinose utilization genes (Madar *et al.*, 2013), initiating the acclimatization of bacterial physiology to the new conditions. The subsequent acceleration of growth is mediated by changes in the amount of active proteins, which is highly regulated. Crucial processes are the production of new proteins, rapid post-transcriptional regulation via mRNA or protein modification, proteolysis and the dilution of old proteins through

cell division (Alon, 2006). Many bacterial proteins are stable i.e. that $\frac{1}{2}$ of their activity is available to the next generation. Stable proteins thereby provide a *phenotype memory* of past environments, which has interesting consequences for rapidly fluctuating environments.

Memory in bacteria

A detailed investigation of cellular memory was performed by Lambert and Kussel for the model-system of lactose utilization in *E. coli* (Jacob & Monod, 1961). Lactose utilization requires expression of the lac operon (yielding LacZ, LacY and LacA), which is repressed by LacI in the absence of lactose (Madigan *et al.*, 2014). Addition of lactose relieves repression via the isomer allolactose that sequesters LacI. The formed lactose utilization proteins are very stable with degradation rates $< 0.6\% \text{ h}^{-1}$ (Mandelstam, 1958; McKenna *et al.*, 1991) so that their concentrations decay mainly by dilution from cell division. Lambert and Kussel induced expression of the lac operon in *E. coli* by growing cells with lactose in a microfluidic device for 4h (Lambert & Kussel, 2014). Thereafter, cells were shifted to glucose for varying amounts of time, before being shifted back to lactose (Figure 1A). Glucose exposure stops expression of the lac operon via cAMP (catabolite repression). Non-induced cells had a lag-time of 38 min, but pre-induced cells continued growth with shorter interruptions (Figure 1B). No lag occurred for intervals ≤ 4 h and significantly shorter lags were measured for intervals up to 12h (Figure 1B), corresponding to 10-12 generations (Lambert & Kussel, 2014). This example is an impressive demonstration of *phenotype memory* that is mediated by stable proteins and can prevent growth delays in fluctuating environments.

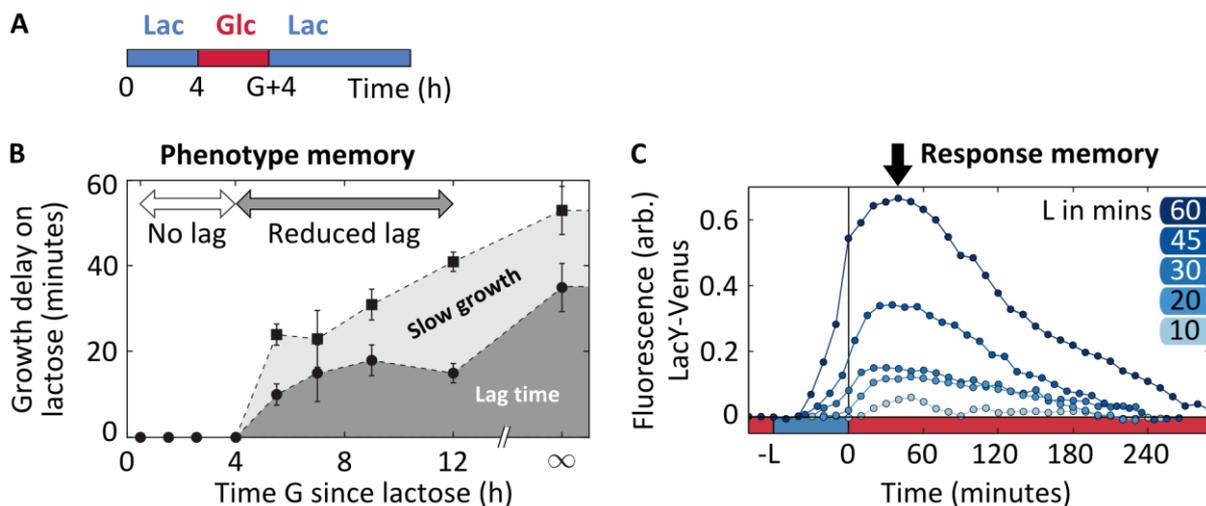


Figure 1. Cellular memory in the lactose response of *E. coli*. (A) Schematic of experiment to measure cellular memory. Two lactose exposures are interrupted by the presence of glucose. (B) Protein stability accelerates response upon second encounter of lactose. (C) Hysteretic response continuation in the absence of lactose. Modified from Lambert & Kussel, 2014.

In the same study system, a second type a memory occurs on finer time scales, i.e. within one generation time: *response memory*, which refers to the hysteretic continuation of response after removal of the stimulus (Lambert & Kussel, 2014). In this example, LacY concentrations continue to increase after a switch to glucose. Decay of protein levels starts 40 -50 min after removal of the inducer (Figure 1C). The overshoot is explained primarily by residual intracellular inducer

(explains 50% of overshoot), but also by binding dynamics of LacI and mRNA stability (Lambert & Kussell, 2014). Response memory protects cellular responses from transient loss of stimulus.

In summary, bacteria physiologically respond to environmental contrasts and responses are stabilized within and between generations. The cellular memory can ensure steady growth in environments with fluctuating resource levels and may further have interesting consequences for the survival in stressful conditions (Lin & Kussell, 2016).

Stress response

Bacteria respond to stress by triggering one or multiple stress-response systems. There are general and specific stress response systems (Storz & Hengge, 2010). General stress response systems target the transcription of a large number of genes via transcription factors and thereby improve survival in many stressful conditions. In *E. coli* the general stress response is regulated by the alternative sigma factor RpoS (sigma 38), which controls the expression of ~ 500 genes (Battesti, Majdalani, & Gottesman, 2011). The expression of RpoS is induced by reduced growth as caused by nutrient limitation, but can also be triggered by rapid changes in acidity, osmolarity, and temperature (Storz & Hengge, 2010). There are a variety of specific stress response systems. *E. coli*, for example, has specific responses against heat shock (36 genes regulated by sigma factors RpoE and RpoH), oxidative stress (~30 genes regulated by activator OxyR), DNA damage (SOS response; ~20 genes regulated by repressor LexA), and several other stressors (Storz & Hengge, 2010; Madigan *et al.*, 2014). There is significant overlap among the targets of stress response systems and their induction signals. Consequently, alternative response systems compete for their activation upon encounter of stress. In *E. coli*, gradual increase of stress induces specific responses, but sudden stress induces general stress response via RpoS (Young, Locke, & Elowitz, 2013). The specificity of the mounted response produces varying degrees of cross-stress protection in fluctuating environments.

In many cases the regulatory overlap of response systems enhances cross-stress survival. For example, heat-shocked *E. coli* had 10-100x higher survival during subsequent acid stress (Wang & Doyle, 1998). Acid survival was likewise increased by previous short pulse of antibiotic treatment as provided by trimethoprim (Mitosch, Rieckh, & Bollenbach, 2017). *Cross-stress protection* can also be mediated by the expression of individual protein complexes. For example, low concentrations of specific antibiotics induce the expression of broad spectrum drug efflux-pumps that transport a wide range of antibiotics and other toxic natural substances (Li, Elkins, & Zgurskaya, 2016). These examples show that pre-adaptation for future environmental stress can be provided by past stress response.

Intriguingly there are opposite cases, where historic stress decreases survival in current stress, a phenomenon called *cross-stress sensitivity*. A well understood example is the NaCl-induced acid sensitivity of *E. coli* that is mediated by expression of the porin PhoE (Rowbury, Goodson, & Humphrey, 1994; Lazim, Humphrey, & Rowbury, 1996). Furthermore, there may be less specific cross-sensitivity interactions as caused by metabolic costs of response memory or even damage from previous stressors. There is comparatively little research on cross-sensitivity, possibly because its existence suggests the infrequency and thus ecological irrelevance of certain transitions in the natural bacterial habitats.

Adaptive response

In addition to active physiological acclimatization, bacteria are also passively adapted to their dynamic environments by the process of natural selection, as originally described by Darwin and Wallace in their joint paper to the Linnean Society in 1858 (Darwin & Wallace, 1858). Due to errors during genome replication, partial genome duplications and lateral gene transfer, genomes are continuously changing (Knöppel, 2016). The genetic variation is linked to variation in fitness, the ability of organisms for translating resources to reproduction. Natural selection optimizes fitness across generations through an ecological sampling process, thereby gradually tuning organisms to their environment (Via & Lande, 1985). Although mechanistically simple, adaptation is a complex process in nature.

Temporal variation broadly selects for reproductive success in the various temporal states of the environment, and the transitions between them. Fitness improvements to the selective components need not be genetically correlated, such that distinct sets of mutations are consecutively selected (*e.g.* Leroi, Lenski, & Bennett, 1994; Jasmin & Kassen, 2007; Kim, Lieberman, & Kishony, 2014). The individually selected mutations can have pleiotropic effects, i.e. that they affect the expression of multiple traits. As a consequence of protein interactions and resource limitations, mutations are nearly always pleiotropic (Wright, 1968; Kacser & Burns, 1981), and a good example for pleiotropy is the evolution of antibiotic resistance in the absence of antibiotics (Rodríguez-Verdugo, Gaut, & Tenailon, 2013; Katz & Hershberg, 2013; Knöppel, Näsvall, & Andersson, 2017). Pleiotropy can generally either cause positive, or negative fitness effects in future environmental states. An example of negative, antagonistic pleiotropy is the generally reduced physiological growth rate of antibiotic resistant bacteria (Andersson & Levin, 1999). In the absence of antibiotic, non-resistant cells outgrow the resistant ones, which can limit the maintenance of resistance after end of treatment (Andersson & Hughes, 2010). Similarly, more frequent environmental fluctuation can interfere with selective sweeps, increase clonal interference, and thereby altogether delay evolutionary dynamics (Harrison *et al.*, 2013). Adaptive response may further be delayed by competing selective pressures, arising from the transitions between environmental states. When transitions entail cross-stress sensitivity, frequent changes may select for optimized shifting. The ensuing competing adaptive priorities are difficult to observe in the wild, but they can be studied efficiently in laboratory.

Evolution experiments

The short generation time and small size of microorganisms enable experimental studies of evolutionary adaptation, on feasible time scales and in controlled laboratory settings. Evolution experiments are easily set up with bacteria. The classic protocol is to grow batch cultures, apply selection during incubation, and serially propagate populations by dilution into fresh media (Kassen, 2014). Samples of the evolving population can be regularly preserved by freezing, to produce a “fossil record” for subsequent characterization. Through measurements of fitness one can infer the rate of adaptive response to selection, as provided by the experimental procedure. In contrast to evolution in the wild, this setup allows for replication and thereby enables the statistical evaluation of pre-formulated hypotheses regarding the dynamics of adaptation (Garland & Rose, 2009). These experiments can test evolutionary theory by asking fundamental questions, such as the following: *Does fitness increase indefinitely? Is adaption gradual or saltatory? Do new functions evolve from random DNA sequences? Does temporal variation affect the rate of adaptation?*

The precise setup of evolutionary experiments is tailored to the questions they address, as illustrated by three examples that take different approaches:

- i) *Parallel adaptation* experiments test whether or not a difference in selection pressure causes distinct adaptive responses in evolving treatment groups. This approach is focused on contemporary comparisons. For example, Lindsey *et al.* investigated how the rate of environmental deterioration affects the dynamics of evolutionary rescue by experimentally treating replicate populations of *E. coli* with increasing concentrations of antibiotic (Lindsey *et al.*, 2013). Treatment groups differed by the rates of concentration increase. Fast increases produced higher extinction and adapted populations had lower genetic diversity, demonstrating that evolution is contingent on the rate of environmental change (Lindsey *et al.*, 2013).
- ii) *Comparative evolution* experiments test the impact of genetic background on the adaptive response to identical selection. For example, Vogwill *et al.* measured parallelism of antibiotic resistance evolution in the *Pseudomonas* clade by subjecting eight species of *Pseudomonas* to selection with rifampin (Vogwill *et al.*, 2014). They discovered that adaptation had pronounced parallelism on the nucleotide level, but that pleiotropic fitness-costs were significantly different between species (Vogwill *et al.*, 2014).
- iii) *Single group evolution* experiments ask fundamental questions concerning adaptation by longitudinal comparisons to the ancestor. The prime example is the long-term evolution experiment that was started by Lenski in 1988 and has lasted > 60 000 bacterial generations (Good *et al.*, 2017). The experiment consists of *E. coli* grown in media containing glucose and citrate. The ancestral strain from 1988 cannot utilize citrate. This experiment has demonstrated that the evolution of key innovations – in this case citrate utilization – requires previous potentiating mutations (Blount, Borland, & Lenski, 2008; Blount *et al.*, 2012), that adaptive specialization frequently involves fitness trade-offs (Travisano & Lenski, 1996), and that the overall adaptive dynamics are characterized by ever-smaller increases in fitness, although they do not plateau (Wiser, Ribeck, & Lenski, 2013).

Experimental evolution contributes to a mechanistic understanding of evolution, as it often focusses on dissecting the underlying constraints that ultimately determine the likely range of evolutionary trajectories.

Adaptive strategies

There are a variety of eco-evolutionary strategies for adaptation to fluctuating selection. In the discussion of potential adaptive responses, I will focus on the early steps of adaptation, as potentially observed in evolution experiments. Two contrasting main strategies are discussed the most by the literature, the emergence of generalist or specialist genotypes, yet there are further adaptive strategies.

Generalists and specialists

A generalist is defined by the expression of one or several traits that increase fitness to multiple temporal states of the environment. The idealized case has equal fitness on all temporal states, which I call a balanced fitness array. A specialist is the opposite strategy, in that a specialist has an unbalanced fitness array being specifically adapted to a single or few temporal states. It is important to note that both definitions are relative to a specific environment; a generalist may be a specialist in other fluctuating environments.

The evolution of generalists is a common adaptive response in rapid and regularly fluctuating environments (Kassen, 2002). Generalists can emerge from single mutations that have positive pleiotropy, or by a combination of mutations. Generalists rapidly evolve in a wide range of fluctuating conditions, as illustrated by an experiment with *P. fluorescens* and selection for growth rate in environments alternating between poor and rich media (Buckling *et al.*, 2007). The study investigated fitness changes during growth in alternating environments with different scales of temporal variation (intervals ranging from 7 to 350 generations), in comparison to control populations evolving in either of the media. Control populations only improved local fitness, and had up to 20% lower fitness in the other media (Buckling *et al.*, 2007). Populations that cycled between media increased fitness in both conditions (Buckling *et al.*, 2007), demonstrating the emergence of cost-free generalists, under a wide-range of temporal scales. There are many more, albeit less systematic, accounts for the rapid evolution of generalists in fluctuating environments (Leroi *et al.*, 1994; Hughes, Cullum, & Bennett, 2007; Ketola *et al.*, 2013; Schenk *et al.*, 2015).

The emergence of specialists is more characteristic for stable environments (Futuyma & Moreno, 1988), yet they may also be selected in fluctuating environments that meet certain criteria. Temporal variation produces niche multiplicity and may thus contribute to the emergence and maintenance of biodiversity (Kassen, 2002), which implies the evolution of specialists due to Gause's law; "*Complete competitors cannot coexist*" (Gause, 1934; Hardin, 1960). Multiplicity of adaptive peaks could be shown for *E. coli* in alternating resource environments, as reflected by increased divergence among replicates, compared to stable environments with either resource or their mixture (Cooper & Lenski, 2010). Yet evidence for the selection of specialists in response to fluctuating selection is rare, and has only been demonstrated for adaptation to targeted selective pressures, as provided by the alternation of the sugars mannose and xylose (Jasmin & Kassen, 2007) or specific pairs of antibiotics (Yoshida *et al.*, 2017). The mechanisms for the selection of single-sided adaptation are not entirely clear, but may result from evolutionary genetic constraints, for which there are indications in both cases.

Reduced responsiveness

Fluctuating environments can also select for altered regulation of cellular responses (i.e. change of phenotypic plasticity). For example, fluctuation may favor the change from inducible responses to their constitutive expression, such that cells effectively skip lag-time – they are pre-adapted (strictly speaking, *pre-acclimated* would be a better term). Such response was selected in *E. coli*, growing in alternating resource environments with glucose and lactose (Cooper & Lenski, 2010; Quan *et al.*, 2012). Constitutive regulation evolved in all replicates (6/6), and had a total frequency of 99.7% among the isolated clones. Constitutive expression also evolved in some replicates, adapting in constant lactose or the mixed environment, yet to a significantly smaller degree (2 or 3/6 replicates; <50% of isolated clones; (Quan *et al.*, 2012). The parallel evolution of pre-adaptation in this setting is likely a special case, because glucose protected from the potential metabolic burden of constitutive *lacZYA* expression – via catabolite repression.

Bet-hedging

Unpredictably changing environments with strong selective pressures may favor the evolution of *bet-hedging* strategies. Bet-hedging refers to adaptive strategies that ensure the survival of populations, by the stochastic production of alternative phenotypes. Although, bet-hedging is a complex phenotype, it can evolve by few genetic changes, as demonstrated in an evolution experiment *P. fluorescens* (Beaumont *et al.*, 2009). Alternating selection for capsulated and non-capsulated cells, selected for novel bistability in the capsule pathway (Gallie *et al.*, 2015); the evolved bet-hedging genotype stochastically produced both cell types, in sectorized colonies (Beaumont *et al.*, 2009). Bistability is common in bacteria; growing cultures naturally produce a fraction of dormant cells, due to stochastic levels of ppGpp (Maisonneuve, Castro-Camargo, & Gerdes, 2013). The dormant sub-population can ensure population survival in stressful environments, *e.g.* because non-growing cells survive antibiotic treatments that are lethal to active cells (Lee, Foley, & Epstein, 1944; Tuomanen *et al.*, 1986). The *persisters* (Bigger, 1944) stochastically revert to normal growth (Balaban, 2004). The frequency of persister cells formation is genetically controlled (Moyed & Bertrand, 1983; Balaban, 2004), but has not been explored using evolutionary experiments.

Elevated mutation

A temporary solution to complex adaptive challenges is the elevation of mutation supply. Elevated mutation rates come at a large cost, because of the rapid accumulation of deleterious mutations. Accordingly, the adaptive benefits of hypermutation are temporary, and there is strong selection for mutation rates to decrease after successful adaptation, as captured by *in vivo* evolutionary experiments of *E. coli* in the mouse gut (Giraud *et al.*, 2001). Interestingly, mutation rate is not equal across the genome, which may be the adaptive solution to this conundrum. There are hyper-variable loci, *e.g.* long stretches of tandem repeats that are prone to frameshift mutations, as caused by slipped strand mispairing (Levinson & Gutman, 1987). It has been postulated that the variation in mutation rate is adaptive, because the mutation hotspots predominately occur in genes that interact with the environment in unpredictable ways (Moxon *et al.*, 1994). These sites were called *contingency loci* (Moxon *et al.*, 1994), due to their putative adaptive value in variable environments. A textbook example is the *ahpC* gene from the oxidative stress response of *E. coli*. *AhpC* contains 4 repeats of TCT in the wildtype (Ritz *et al.*, 2001). Peroxidase function is frequently lost, due to the reversible addition of a 5th TCT repeat. The extra repeat is a gain-of function mutation that converts enzyme function from peroxidase to a disulfide reductase (Ritz *et al.*, 2001). A genetic switch reminiscent of a contingency locus, was experimentally selected in *P. fluorescens* by selection for alternating production of biofilm (Hammerschmidt *et al.*, 2014). The most successful genotype had a *mutS* mutation that generally elevated mutation rate, yet as one of the genes controlling biofilm formation had a tract of 7 Gs in the active site, the *mutS* mutation actually converted that gene into a genetic switch. Reversible addition of a single G resulted in reliable phenotype switching (Hammerschmidt *et al.*, 2014).

Altogether, the evolution of generalists seems to be ultimate adaptive response to fluctuating selection. Specialists and other more complex strategies are only maintained under specific conditions. Their emergence and maintenance may, therefore, be indicative of stronger adaptive constraints that prevent the evolutionary modification of traits from reaching an adaptive optimum; they either slow the approach of the optimum, or shift adaptation away from the optimum (Hansen, 2015). Initially selected strategies may eventually be outcompeted by others, or even transform. For example, a specialist can turn into a generalist by additional mutation.

Finally, adaptation to fluctuating environments may proceed via unknown adaptive strategies, adding elements of suspense and surprise to every evolutionary experiment.

Objectives

In this thesis I experimentally study, how temporal variation affects the adaptive response of bacteria in a novel fluctuating environment. My general aim is to contribute to the understanding of adaptive constraints in changing environments. I approach this aim with laboratory evolution experiments that investigate the following specific aims:

Influence of different fluctuation characteristics on adaptive dynamics

We are only beginning to understand how different flavors of temporal variation affect evolutionary dynamics. With the approach of parallel evolution I investigate the quantitative impacts of temporal regularity, fluctuation interval, breadth of selective pressure, and fluctuation order on rates of adaptation.

Evolutionary stability of adaptive constraints

Antagonistic pleiotropy frequently emerges as a result of directional selection. Evolution in fluctuating environments may be limited by these genetic fitness trade-offs. This opens the question as for their stability, i.e. whether they preclude the reaching of an adaptive optimum, or whether populations can escape the constraint via alternative evolutionary trajectories. Using the approach of comparative evolution, I test the evolutionary stability of reciprocal genetic trade-offs.

Importance of phenotypic memory

The impact of bacterial phenotypic memory for fitness optimization in rapidly fluctuating environments has hardly been investigated. I hypothesize that phenotypic memory can facilitate, or constrain adaptation, by changing selection pressure at environmental transitions. Cross-stress protection may buffer the fitness effects of antagonistic pleiotropy. Cross-stress sensitivity may itself be a competing selective force. I investigate how changes in fluctuation frequency, and thus the occurrence of phenotypic memory, affect evolutionary adaptive dynamics.

Application of findings to limit resistance evolution

Evolution is not a historic account, but an active process that increasingly affects our society. The prime example is the spread of antibiotic resistance in response to the use of antibiotics. A key motivation for this thesis is to test, whether we can apply evolutionary principles to limit the emergence of antibiotic resistance. The model system is carefully selected to facilitate translation of findings to applications in antibiotic therapy.

Model system

The model system for my evolutionary investigations is the bacterium *Pseudomonas aeruginosa* and fluctuating selection, as provided by the sequential exposure to antibiotics. Antibiotics provide focused selective pressure with described mechanisms of action. Moreover, the genetics of the adaptive response – antibiotic resistance – are well studied. The identification of selection regimes that inhibit resistance evolution implies ways to stabilize effective antibiotic therapy. This aim is supported by the choice of *P. aeruginosa* as a model organism, due to its pathogenicity and high potential for resistance evolution.

Antibiotics

Antibiotics are molecules that kill or inhibit the growth of bacteria and fungi. The antibiotics used for therapy today are mostly derived from naturally occurring bioactive compounds that are produced by certain fungi and bacteria, most notably actinomycetes. Many antibiotics, therefore, have natural functions in the ecology of bacteria, notably microbial antagonism (Roberts, 1874; Tyndall, 1876; Waksman & Woodruff, 1940; Currie *et al.*, 1999) and signaling (Hopwood, 1981; Linares *et al.*, 2006). Other antibiotics are of synthetic origin. The discovery of antibiotics (Ehrlich, 1911; Fleming, 1929; Klee & Römer, 1935) transformed medicine, as they enabled treatments for previously untreatable infections. Extensive research efforts in the 1940s and 1950s – motivated by treatment success and the political situation – yielded an impressive arsenal of antibiotics.

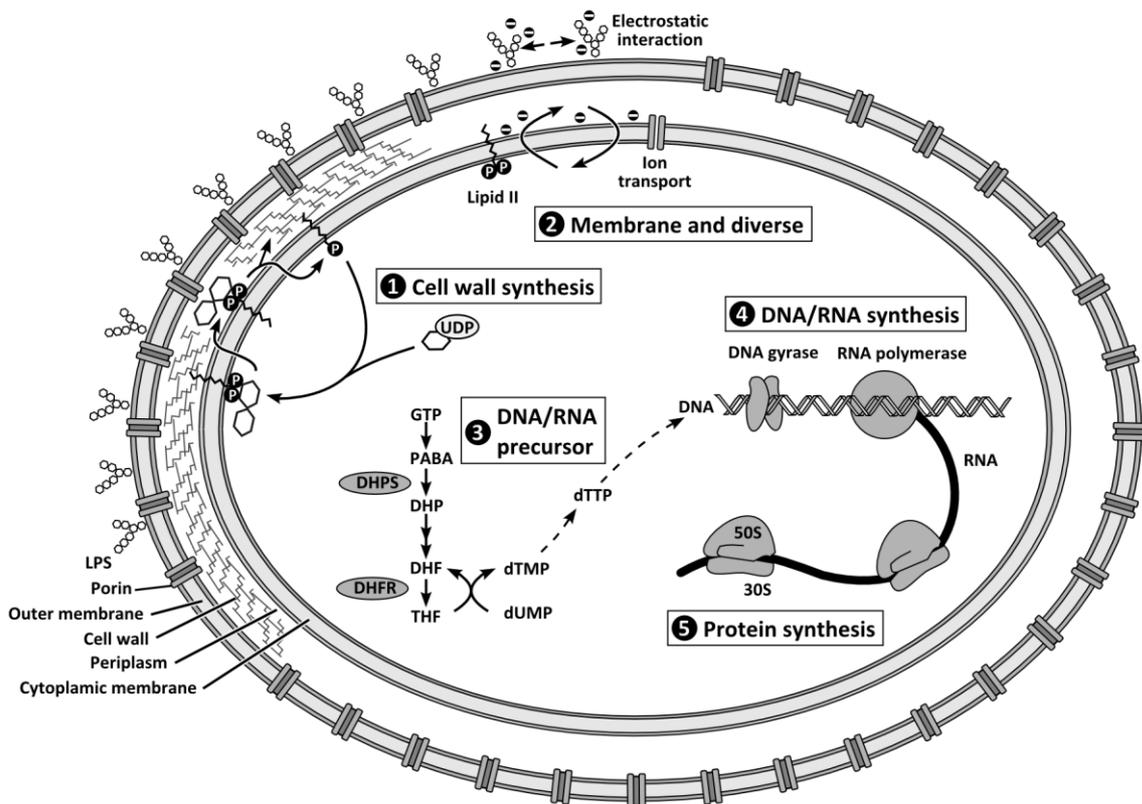


Figure 2. Cellular targets of antibiotics in Gram-negative bacteria. DHPS, dihydropteroate synthase; DHFR, dihydrofolate reductase; DHP, dihydropteroate; LPS, lipopolysaccharide; PABA, para-aminobenzoic acid; dTMP, deoxythymidine monophosphate; dTTP, deoxythymidine triphosphate; dUMP, deoxyuridine monophosphate; UDP, uridine diphosphate. Modified from Walsh, 2003.

Antibiotics are grouped into functional classes according to their cellular targets. Antibiotics of the same class have similar chemical structures (Walsh, 2003). Most antibiotics target one of several major targets (Figure 3), notably: cell-wall synthesis (β -lactams), protein synthesis (aminoglycosides, macrolides, tetracyclins), DNA or RNA synthesis (quinolones, rifampin), or the folate biosynthesis pathway that synthesizes precursors for DNA and RNA synthesis (trimethoprim, sulfamethoxazole) (Walsh, 2003). The most relevant minor targets are membranes (daptomycin, polymyxin) and mycolic acid synthesis (isoniazid). It is increasingly clear that some compounds have multiple targets, as is the case for aminoglycosides (Davis, 1987) and certain antimicrobial peptides (Andersson, Hughes, & Kubicek-Sutherland, 2016).

Antibiotic resistance

The success of antibiotics prompts their lavish use, causing massive leakage into natural environments via waste water from the pharmaceutical industry, hospitals, homes and, most regrettably, agriculture. Low concentration of antibiotics elicit multitudinous responses in microorganisms (Andersson & Hughes, 2014) and can select for the emergence of clinically relevant resistance mutations (Gullberg *et al.*, 2011). There are also pre-existing, ancient resistance genes in the bacterial pangenome (*e.g.* the vancomycin operon *vanHAX*), whose natural function we do not know, but that are now causing major challenges for therapy due to their dispersal on mobile genetic elements (D'Costa *et al.*, 2011). Consequently, in a worrying analogy to an evolution experiment, antibiotic resistance is emerging and spreading globally (Laxminarayan *et al.*, 2013).

Antibiotic resistance can be achieved by three general mechanisms (Walsh, 2003):

- i) modification of target structures that affect antibiotic binding dynamics,
- ii) modification of target access by changing antibiotic entry or expulsion,
- iii) enzymatic breakdown of the antibiotic.

Target modifications and de-repression of efflux pumps of β -lactamases is often mediated by single nucleotide changes called SNPs (single nucleotide polymorphisms) in the DNA that mostly occur due to errors in DNA replication. In most bacteria, per generation mutation rates are in the range of 10^{-10} - 10^{-9} per site (Lynch *et al.*, 2016), translating to 10^{-8} to 10^{-7} per gene. An approach to thwart resistance evolution is to decrease population size to level at which *de-novo* evolution of resistance is highly unlikely. However, there are alternative mechanisms that may serve as stepping-stones to resistance, such as gene amplification, and tolerance. Partial genome amplifications that increase the copy number of genomic regions can be very frequent in populations of particular pathogens, in the range of 10^{-5} to 10^{-2} per gene and generation as measured in *Salmonella enterica* (Anderson & Roth, 1981). For example, the partial genome duplication spanning the drug efflux pump AcrAB conferred rapid adaptive response to combination treatment with the antibiotics erythromycin and doxycycline in *E. coli* (Pena-Miller *et al.*, 2013). The amplifications are highly unstable and are rapidly lost upon removal of selective pressure (Laehnemann *et al.*, 2014). It is likely that partial genome duplications frequently occur during antibiotic therapy, as they are a molecular mechanism for *heteroresistance* (Hjort, Nicoloff, & Andersson, 2016), the commonly observed microbiological phenomenon of mixed resistance profiles in clinical isolates.

Antibiotic tolerance is a bacterial survival strategy that relies on the reduced bactericidal activity of antibiotics, against slowly growing cells (Lee *et al.*, 1944; Tuomanen *et al.*, 1986). Tolerant mutants do not have increased resistance, as reflected in unchanged minimal inhibitors

concentrations (MIC), but their low physiological growth rate decreases the killing rate of antibiotics compared to susceptible strains (Brauner *et al.*, 2016). Tolerant strains can be selected by short exposures to high drug concentrations (Fridman *et al.*, 2014; Levin-Reisman *et al.*, 2017).

Importantly, the expression of resistance is modulated by environmental and physiological contexts, as recently reviewed (Hughes & Andersson, 2017). An illustrative example is the expression of mecillinam resistance as caused by *cysB* mutation in *E. coli*. The addition of cysteine completely suppresses the expression of resistance from *cysB* mutations, which otherwise cause >100-fold increase in resistance (Thulin, Sundqvist, & Andersson, 2015). The expression of resistance by the same mutation is also modulated by the osmolarity in urine, suggesting a possibility to influence treatment success by drinking more water or supplementing nutrition with cysteine (Thulin, Thulin, & Andersson, 2017).

Antibiotic resistance is a pleiotropic trait. Bacteria selected for resistance against single antibiotics usually display cross-resistance to antibiotics of the same class (Szybalski & Bryson, 1952; Imamovic & Sommer, 2013; Lazar *et al.*, 2014; Barbosa *et al.*, 2017; Imamovic *et al.*, 2018), which would translate to fitness benefits at sequential encounters of these drugs. The same resistance mutations can also have negative fitness effects, so called collateral sensitivity, during treatment with other antibiotics. It remains to be tested how these trade-offs affect the maintenance of antibiotic resistance in clinical and natural environments.

Pseudomonas aeruginosa

P. aeruginosa is a motile, rod-shaped γ -proteobacterium (Gram-negative) that characteristically produces green pigments in liquid culture (Madigan *et al.*, 2014). Its metabolic versatility enables growth on unusual carbon sources and *P. aeruginosa* can be easily isolated from soil and water (Ramos, 2004). Importantly, *P. aeruginosa* is also an opportunistic pathogen with broad host range causing virulence in plants, invertebrates but also in humans (Rahme *et al.*, 1995).

Infections by *P. aeruginosa* frequently occur in the lungs of cystic fibrosis (CF) patients, where the bacteria dwell in sticky mucus. The mucus is a consequence of a dysfunctional ion-transport in the lung epithelium of the patients as caused by mutations in the cystic fibrosis transmembrane regulator CFTR (Winstanley, O'Brien, & Brockhurst, 2016). Bacterial infections are the primary cause of mortality in CF patients and extensive chemotherapy is employed to prevent exacerbations in bacterial density. Therapy is usually focused on *P. aeruginosa* and involves more or less constant selective pressure by antibiotics (Döring *et al.*, 2012), as illustrated by a cohort of chronically *Pseudomonas*-positive patients that receive regular prophylactic treatments at the Universitätsklinikum Schleswig-Holstein in Kiel. Patients in this cohort regularly inhale tobramycin, colistin or aztreonam at home (Tüffers, 2018). They occasionally receive 2-week courses of prophylactic intravenous treatment with combinations of two, sometimes three drugs, during which some patients choose to continue their inhalations (Tüffers, 2018). The constant antibiotic exposure is a potent selective pressure and *P. aeruginosa* undergoes rapid adaptive evolution within patients, in response to drug treatment (Winstanley *et al.*, 2016). Phenotypic changes between longitudinal isolates are commonly the evolution of mucoid phenotypes and increases in antibiotic resistance (Damkiær *et al.*, 2013; Marvig *et al.*, 2015). *P. aeruginosa* has an impressive array of chromosomally encoded efflux pumps (Li *et al.*, 2016) enabling rapid evolution of multidrug resistance via regulatory changes, such as mutational inactivation of efflux repressors (Breidenstein, de la Fuente-Núñez, & Hancock, 2011). It is, therefore, not surprising that certain strains of *P. aeruginosa* were recently ranked as the second most critical resistance

threat by the World Health Organization (World Health Organization, 2017). Altogether, *P. aeruginosa* is a good model for the development of new antibiotic treatment strategies.

Thesis content

Chapter 1: Evolutionary ecology meets the antibiotic crisis: Can we control evolution? Roderich Römhild and Hinrich Schulenburg. – Manuscript submitted for publication

In this opinion paper, we reviewed common antibiotic treatment practices, from the perspective of evolutionary ecology. We argued that principles from ecology and evolution may contribute to the design of more sustainable treatments that inhibit the emergence of resistance. Recent and neglected older investigations supported a re-consideration of sequential treatments. We proposed evolution experiments as an efficient tool for the investigation of new treatment strategies. We further discussed the advantages of antibiotics as a laboratory model system for the study of evolutionary dynamics.

Chapter 2: Adaptive paths to escape collateral sensitivity cycling. Roderich Römhild*, Camilo Barbosa*, Philip Rosenstiel, and Hinrich Schulenburg. – Manuscript

We investigated the evolutionary stability of genetic constraints, as provided by two cases of reciprocal collateral sensitivity. We performed comparative evolution starting with characterized resistant clones, which were subjected to increasing concentrations of antibiotics. We specifically investigated how new mutations selected by the second antibiotic affected the expression of previous resistance phenotypes. The main finding was that reciprocal collateral sensitivity generally constrains the evolution of dual resistance, but that rare mutations can enable bacteria to escape the genetic trade-off. The probability of escape was dependent on the order of antibiotic selection. To our knowledge, this is the first experimental test for the efficacy of collateral sensitivity cycling. *This manuscript has joint first-authors.

Chapter 3: Negative hysteresis improves antibiotic cycling efficacy. Roderich Römhild, Chaitanya S. Gokhale, Christopher Blake, Philip Rosenstiel, Arne Traulsen, Dan I. Andersson, and Hinrich Schulenburg. – Manuscript submitted for publication

The overall aim of this study was to gain a systematic insight into fitness optimization and adaptive priorities in fluctuating environments. We performed parallel evolution experiments with selection by 16 different sequences of three bactericidal antibiotics (ciprofloxacin, gentamicin, and carbenicillin). The sequences differed with respect to drug order, switching rate, and temporal regularity. The antibiotics were chosen for their association with collateral sensitivity and cross-stress sensitivity (so-called *negative antibiotic hysteresis*). The main finding was that physiological balance is an important driving force in bacterial evolution.

The cumulative influence of negative hysteresis accurately predicted rates of resistance evolution, across the treatment sequences. The correlation was explained by a genetic trade-off between resistance and a novel response, insensitivity to hysteresis, which was mediated by mutations in *cpxS* (formerly known as PA3206 or PA14_22730). Experimental change of hysteresis levels in follow-up experiments predictably altered rates of resistance evolution, as evaluated by population extinction and resistance gains. Our results indicate new ways to improve treatment efficacy – sequential protocols with high hysteresis density that select for physiological balance and thus inhibit the evolution of resistance.

Chapter 4: Sequential treatment with three β -lactams in *Pseudomonas aeruginosa* and the evolution of resistance. Roderich Römhild and Hinrich Schulenburg. – Manuscript

The aim of this study was to investigate the dynamics of resistance evolution during treatment with antibiotic that individually inhibit cell wall synthesis (carbenicillin, cefsulodin, and doripenem). The performed parallel evolution experiment had identical setup to the main experiment of Chapter 3, but the antibiotics displayed cross-stress protection. As a result, populations evolved resistance more rapidly. However, fast-switching treatments significantly increased the likelihood of population extinction. The observation seemed to be explained by uncorrelated genetic responses and the evolution of collateral sensitivity within β -lactams. Our observations challenge the notion that multidrug treatments with similar antibiotics are ineffectual.

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Chapter 1

Manuscript, submitted as opinion article to *Trends in Ecology and Evolution*

Evolutionary ecology meets the antibiotic crisis: Can we control evolution?

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Abstract

The spread of antibiotic resistance is a global challenge that is fueled by evolution and ecological processes. We here argue that principles from evolutionary ecology can be applied to control the emergence and spread of resistance. We specifically propose that pathogen adaptation can effectively be constrained by temporal variation, especially when changes are fast and/or irregular and combined with genetic and physiological trade-offs in the evolving organisms. We then outline how work on antibiotic resistance can simultaneously advance a mechanistic understanding of evolution, as it often focusses on dissecting the underlying constraints that ultimately determine the likely range of evolutionary trajectories. We conclude that it is high time for more evolutionary ecologists to get involved in antibiotic research.

Main text

Antibiotics and antibiotic resistance are an ancient part of bacterial ecology [1–3]. In the face of the current antibiotic crisis, we should therefore remember that antibiotic resistance is a pleiotropic trait that usually entails ecological trade-offs [4] (see Glossary). As a consequence, we can apply principles from evolutionary ecology to improve our treatment protocols with the aim to constrain the emergence of drug resistances. At the same time, resistance evolution shows very high potential to enhance our general understanding of adaptation, because it can be easily studied with controlled laboratory-based evolution experiments, because comprehensive reference data sets are already available from *in vitro* and also *in vivo* studies, and because it allows us to connect ecological factors to evolutionary processes and also the underlying molecular and genetic mechanisms. The selective pressure (antibiotics), the evolving organisms (bacteria) and the evolutionary genetics of adaptation (space of resistance mutations and the distribution of their fitness effects) are often well characterized in this system. Intriguingly the eco-evolutionary feedbacks, which may drive resistance evolution, are not always well

understood and are usually neglected in this field of research – in spite of their potential importance.

With this opinion paper, we highlight how concepts based in evolutionary ecology may yield novel ideas for antibiotic therapy. We identify sequential antibiotic treatments as a highly potent treatment option, which we believe should make it difficult for bacteria to adapt because of the continuously changing selective challenge and which is usually not considered for therapy. Surprisingly, we currently possess only comparatively little detailed knowledge on how organisms adapt to such rapidly fluctuating environments. Therefore, the research that we propose should also help to enhance our general understanding of the processes and mechanisms that underlie adaptation to temporarily variable conditions. In the following, we will show that current procedures of antibiotic treatment are sub-optimal from the view of evolutionary ecology (Section 1). We then discuss ecological principles that may improve treatment sustainability (Section 2). We conclude the paper, by outlining how selection experiments with antibiotics enable evolutionary ecologists to gain a mechanistic understanding of adaptation (Section 3).

Section 1: Sub-optimality of common treatments

Historically, the first strategy for antibiotic therapy was to treat patients for several days with an antibiotic, typically of broad-range activity, such as penicillin. Such monotherapies are still the main treatment form today, yet resistance to the single drugs can evolve rapidly through natural selection [5]. Fast adaptation to individual antibiotics is usually caused by three main non-exclusive factors: (i) a high number of different mutations can confer resistance and these may easily arise due to usually large bacterial population sizes and/or horizontal gene transfer, (ii) the selective advantage of any resistance mutations is large, even if originally rare, and thus they can spread fast through the population; and (iii) non-resistant competitors are excluded by the action of the antibiotic, further enhancing proliferation of the resistant varieties (i.e., competitive release). Evolutionary biologists seek ways to prevent the rapid fixation of resistance mutations by limiting these processes. One approach is to increase the complexity of the environments – and their adaptive landscapes – by applying several different drugs within a single treatment [6]. It is more likely for bacteria to become resistant to a single drug than to several drugs, because there are fewer mutations that provide cross-resistance. These drugs can be deployed simultaneously or consecutively (Fig. 1). It is important to differentiate the hierarchical level at which multidrug treatments are implemented, i.e. with focus on patient groups or individuals (Fig. 1). The approaches have different rationales: Group level application (hospital, cohort, intensive care unit) aims at limiting the spread of resistance caused by cross-infection. Application in single patients aims at the prevention of the emergence of resistance during treatment.

Simultaneous multidrug treatment of patient groups is termed *mixing* therapy [7]. Within an intensive care unit (ICU) multiple antibiotics are applied the same day, but patients individually only receive a single drug (Fig. 1A). Throughout the whole treatment, medication of a patient remains constant, such that each patient effectively receives monotherapy. This strategy produces a patchy selective environment and thus increases spatial but not temporal variation. Therefore the likelihood of *de-novo* resistance evolution in a single patient is not decreased over monotherapy.

Combinations of two or more drugs within the same patient (Fig. 1B) produce more complex adaptive landscapes due to drug interaction. Drug interaction can provide immediate advantage

if drugs synergistically enhance their inhibitory effect on bacterial growth. Certain antibiotic combinations have therefore been used to combat infections fast and efficiently [8] and combination treatment is now the standard for several bacterial infections [9,10]. However, simultaneous drug deployment was repeatedly observed to accelerate evolutionary rescue *in vitro* [11–13]. Resistance evolved earlier in experimental populations treated with combinations than in populations treated with monotherapy, because aggressive treatments release rare multidrug resistant variants from competition with non-resistant cells. Simultaneous treatments may therefore rather obstruct the intended clearance of pathogens. This may explain, why clinical trials failed to show a general advantage in patient recovery and survival after combination therapy as compared to monotherapy [14]. The continued interest in combination therapy is partly due to discoveries of special drug combinations with suppressive interaction [15,16]. These combinations can limit bacterial resistance evolution by selecting against mono-resistant mutants in a specific concentration window. Yet, these drug pairs need to be applied in higher doses than in monotherapies, potentially causing stronger side-effects [6]. Altogether, we suggest to reconsider sequential drug protocols as an alternative treatment strategy, as it may unite the benefits of combination therapy with sustainability, due to additional adaptive constraints caused by the temporal complexity.

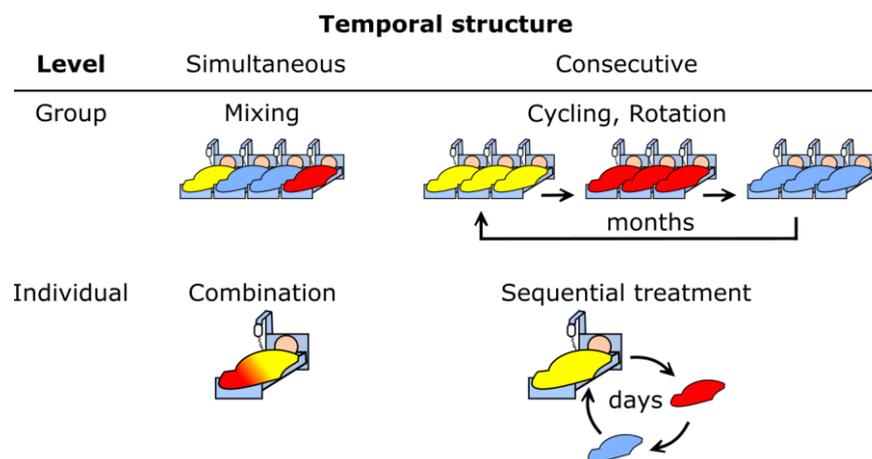


Figure 1. Strategies for multidrug treatments. Multidrug treatments can be designed in different ways, depending on the temporal structure and the application level. Colours represent different drugs.

To date, the idea of sequential treatment has been applied mostly on the group level. In *rotation* or *cycling* therapy the whole ICU is treated with the same antibiotic, which is periodically switched for a new antibiotic after several weeks (Fig. 1C). As switching interval is longer than hospital stay, the likelihood of resistance emergence is not reduced compared to monotherapy. A recent meta-analysis of clinical trials for cycling therapy could show an overall benefit compared to mixing [17] but this effect was due to a reduced number of hospital acquired infections and not because selection for resistance was minimized [18]. We argue that sequential therapy can minimize resistance evolution, but not when it is carried out with the currently used unit-wide approach and the long switching intervals. Drug resistance evolves within single patients (*e.g. Mycobacterium tuberculosis* [5], *Pseudomonas aeruginosa* [19]). To limit the emergence of resistance, multidrug treatments have to be applied to one patient, such that they potentially affect a single population of pathogen. To achieve this aim, drugs need to be rotated more rapidly

than in the unit-wide protocol, i.e. each day (Fig. 1D) or more often. Frequent switching produces fluctuating selection to which adaptation is more difficult. Any particular switch of antibiotics during treatment may improve treatment outcome by curing strains resistant to the preceding antibiotic [17]. Clinical trials on fast sequential treatments proved effective against *Helicobacter pylori* infections [20]. Likewise, sequential therapy increased eradication of *P. aeruginosa* in a small cohort of cystic fibrosis (CF) patients [21]. Intriguingly, the latter study was already published 30 years ago in the Lancet, but did not receive any attention (less than 10 citations within the 30 years according to Web of Science).

Section 2: Controlling resistance emergence by temporal variation

Sequential treatments complicate adaptation because they produce dynamically changing adaptive landscapes for pathogen populations. The selection dynamics can be optimized according to eco-evolutionary principles. We argue that the full potential of sequential treatments can be achieved by considering a) pleiotropic fitness effects of resistance mutations, b) physiological interactions that occur at switches between drugs, c) a sufficient rate of environmental change, and d) sequence stochasticity.

a) Antagonistic pleiotropy

Most proteins are part of interconnected biological networks. As a consequence, adaptive mutations nearly always affect the expression of multiple traits (i.e., pleiotropic effects; [22,23]). Adaptive mutations are therefore often associated with fitness trade-offs in distinct environments [24–27]. In the context of antibiotic treatment, switching drugs in a certain way can potentiate treatment and re-sensitize bacteria due to the antagonistic pleiotropy of previous resistance mutations.

The importance of pleiotropy for the evolution of resistance has recently been reinforced by the rediscovery of the concept of collateral sensitivity, originally introduced more than 60 years ago (Box 1). The evolution of resistance to one antibiotic can increase susceptibility to antibiotics of other classes. The published sensitivity maps [28–32] show antibiotic class specific patterns, which indicates that collateral sensitivity originates from constraints caused by the general *Bauplan*, i.e. structural architecture [33] of the cell. Indeed, genetic investigations confirmed that collateral sensitivity can result from resistance mutations against a first drug that simultaneously enhances uptake of a second antibiotic. For example, collateral-sensitivity was found for strains of *Escherichia coli* adapted to aminoglycoside antibiotics [28–30]. Resistance against aminoglycosides is often caused by mutations that decrease membrane potential, for example by targeting the K⁺-ion-transporter TrkH [29,30,34]. This reduces the uptake of aminoglycosides [35] but also impedes the efficacy of drug efflux pumps such as AcrAB [29], thereby constraining the cellular removal of other drugs, causing hyper-sensitivity. A similar phenotype is achieved by alternative mechanisms in *P. aeruginosa*. Fluoroquinolone-resistant strains of *P. aeruginosa* frequently show collateral sensitivity to aminoglycosides and β -lactams [31,32,36], which is caused by mutations that alter the expression of efflux pumps, e.g. via mutation of *nfxB* [37], the major transcriptional repressor of the multidrug efflux pump MexCD-OprJ [38], or other efflux regulators such as *mexZ* or *nalC* [31]. The resulting changes in expression of particular efflux pumps however affects expression of alternative pumps [38], suggesting that collateral sensitivity is caused in these cases by a deviation from natural efflux balance.

Recent experimental tests of sequential treatments that involve collateral sensitivity highlight their potential application in therapy. Evolved *P. aeruginosa* strains that acquired resistance against the β -lactam piperacillin during treatment, could be re-sensitized by switching to ciprofloxacin [36], possibly due to *nfxB*-mediated changes in pump expression. Rapid alternating treatments of *E. coli* with drug pairs involving the antibiotic polymyxin resulted in one-sided adaptation and thus the suppression of resistance emergence to one of the drugs [39]. Although the mechanism is not entirely clear, it is likely associated with collateral sensitivity.

A second, more general case of pleiotropy is the usually reduced growth rate of antibiotic resistant mutants, which can result from sub-optimal metabolic flux. The reduced growth rate of resistant mutants is often called a *fitness cost* [40] because it increases competition with non-resistant types and this clonal interference can decelerate adaptation [41].

b) Negative physiological interactions

Bacteria physiologically respond to stress, as caused by antibiotics, by activating stress-response systems that alter transcription of a large number of genes and thereby improve survival for the current conditions [42]. Because many bacterial proteins are stable, induced responses can be phenotypically inherited [43] and may thereby provide cross-stress protection to new conditions. Intriguingly, there are also cases where the previously experienced stressor decreases survival in new stressful environment, a phenomenon called cross-stress sensitivity. A comparatively well understood example is NaCl-induced acid sensitivity in *E. coli*, which is mediated by expression of the porin PhoE [44,45]. Furthermore, there may be less specific cross-sensitivity caused by a metabolic cost of hysteretic response memory [46] or directly by stress-induced damage. Antibiotics themselves can induce responses that entail fitness disadvantages when drugs are switched in sequential treatments. Again, the ecological phenomenon itself was already studied 50 years ago, but has since received negligible attention: Sub-lethal pre-treatments with β -lactam antibiotics potentiate killing on aminoglycoside antibiotics in several species of bacteria [47,48] (Box 2). Such physiological potentiation can help to eradicate chronic infections, as demonstrated experimentally (Box 2) or indicated by the high efficacy of sequential protocols in the treatment of biofilms [49]. It remains to be seen whether or not physiological interactions, in addition to their immediate therapeutic benefits, influence resistance evolution, for instance by shifting the priority of adaptation from resistance towards overcoming the physiological transitions.

c) Frequency of change

Fluctuating selection can delay adaptation, because it interrupts selective sweeps. For example, rapid but not slow fluctuation in media quality prevented co-evolution between bacteria and phage [50]. Likewise, switching rate determines the evolvability in the case of antibiotics. If antibiotics are switched too slowly in a sequential protocol, resistance mutations spread through the population, as in monotherapy. In contrast, more rapid fluctuations, such as switching antibiotics every 12 h or 24 h, can limit resistance evolution, as recently demonstrated for the pathogens *P. aeruginosa* [51] and *Staphylococcus aureus* [52] using experimental evolution. Interestingly, these experiments used sub-lethal antibiotic concentrations and achieved both a deceleration of adaptation and also increased population extinction [51]. The latter is likely explained by the increased occurrence of selection pulses as caused by physiological interactions and genetic trade-offs.

d) Stochasticity

Unpredictably occurring environmental disturbances are more difficult to adapt to than regularly occurring selective pressures [53,54]. According to the hypothesis of environmental adaptive conditioning [55] – the terminology is an analogy to associative learning [56] – evolution adjusts gene expression to regular patterns of stimuli. Correlated environmental factors are a common feature of microbial habitats and several microbes exhibit anticipatory gene regulation [57,58]. These organisms use trigger molecules in their environment to adjust gene regulation for future challenges. One example is *Vibrio cholera*, which during the last phase of the infection of the human intestine already induces genes necessary for survival in the aquatic environment outside the host [59]. Anticipation was likewise selected by the fixed sequential contrasts in the human gut. Following transmission, *E. coli* encounters lactose in the proximal part of the intestine, followed by maltose in the distal part three hours later [60]. In the scramble for nutrients, *E. coli* benefits from up-regulating maltose-metabolizing genes ahead of time (lactose induces expression of the maltose operon), thereby skipping the lag-phase associated with the shift in carbon sources [55]. The anticipatory regulation and its fitness advantage are lost when wildtype *E. coli* were grown in constant lactose environment in the lab, indicating a cost of the anticipation behavior [55]. A mathematical model predicts the evolution of anticipation under certain conditions: strong temporal correlation of stimuli, short time between stimuli, and high benefit of the anticipation [57]. These examples illustrate that predictable patterns in sequential antibiotic therapy are potentially dangerous, because they generate the parameter space for the evolution of anticipation. The ensuing adaptive response may be circumvented by irregular drug orders.

Aside from limiting fitness benefits of anticipation, stochasticity in fluctuations can also directly decelerate adaptation. This was demonstrated with populations of viruses, which were exposed to regularly alternating and randomly changing temperatures [61]. In contrast to the observed fitness increases in regularly alternating environments, unpredictable temperature fluctuations led to a significant decrease of fitness [61]. Similarly, fitness returns of bacteria adapting to randomly fluctuating pH were lower than those attained in regularly alternating sequences of pH [62]. The incorporation of temporal stochasticity in sequential protocols may thus additionally restrict resistance evolution in the long-term. We expect the decelerating effect of randomness to increase with the total number of drugs, because of the exponential increase in the number of possible switching directions ($N = x!$). The potential for stochastic orders to decelerate adaptation is mostly unexplored, as trials for sequential treatments with random orders have focused on drug pairs [13,51].

Altogether, principles from evolutionary ecology can be tested for their ability to slow down the emergence of resistance using laboratory experiments. The data thus far generated indicate that complex treatments such as combination or sequential treatments limit resistance evolution. Nevertheless, bacteria may ultimately be able to adapt to treatment by their enormous evolutionary potential, even if only rare evolutionary trajectories are available [63]. Bacteria use genetic loop holes, notably cross-resistance and phenotypic heterogeneity, to escape treatment. The likelihood of cross-resistance strongly depends on the choice of antibiotics. Ideally, the antibiotics select from distinct sets of beneficial mutations. A first step towards this goal is to choose drugs that target different cellular functions, because cross-resistance is particularly common within drug classes, although there are noteworthy exceptions due to epistasis, in particular with β -lactams [64,65]. Bacteria can also adapt to unpredictable disturbances by

increasing phenotypic heterogeneity [66], which is produced by stochastic noise in gene expression [66,67]. The variability in gene expression is often linked to variability in antibiotic tolerance [68–70], which is explained by growth rate dependent killing [71,72]. A certain frequency of dormant cells, so called persisters, is naturally produced by stochastic partitioning of proteins after cell division [73,74] and represents an ancient evolutionary survival strategy; bet-hedging. Rather than relying on the phenotype of highest fitness in the current environment, bacteria *spread their eggs* (resources) *across several baskets* (phenotypes) to reduce the risk of losing them all at once. Phenotypic heterogeneity may thus be an adaptive strategy for unpredictable antibiotic treatments.

Section 3: Opportunities for ecologists in antibiotic research

Opportunities for ecologists arise from the well-studied genetics of antibiotic resistance to learn about evolution. Antibiotics can be used to test evolutionary theory in evolution experiments across short time frames – because antibiotics can provide strong selective pressure – and well-controlled selective conditions – because antibiotic selection precisely targets certain cellular functions and inhibition levels are controlled by concentration. We argue that more can be learned about evolution through the study of adaptive constraints rather than the adaptations themselves. For example, experiments with antibiotics have helped to understand the ecological parameter space that favours the evolution of specialists over generalists in fluctuating environments. Theory predicts the evolution of broad niche width in response to fluctuation [75] and this has been observed in many cases [76], making it mechanistically interesting to see cases for the evolution of specialists. In the experiments, specialists evolved in response to targeted selection and when adaptation invokes pleiotropic trade-offs [39,77], as provided by specific sugars or antibiotics. This contrasts with the experimental evolution of generalists in response to less specific selective pressures, such as temperature fluctuations or patch quality that select towards general stress-response and associated pre-adaptation to other stressors [78,79]. Similarly, a whole suite of questions that would be hard to tackle using less controllable or less studied systems, can be addressed using antibiotic selection. Some intriguing questions arise from the points presented in this paper (Box 3). We conclude that antibiotics are a practical toolbox for evolutionary research.

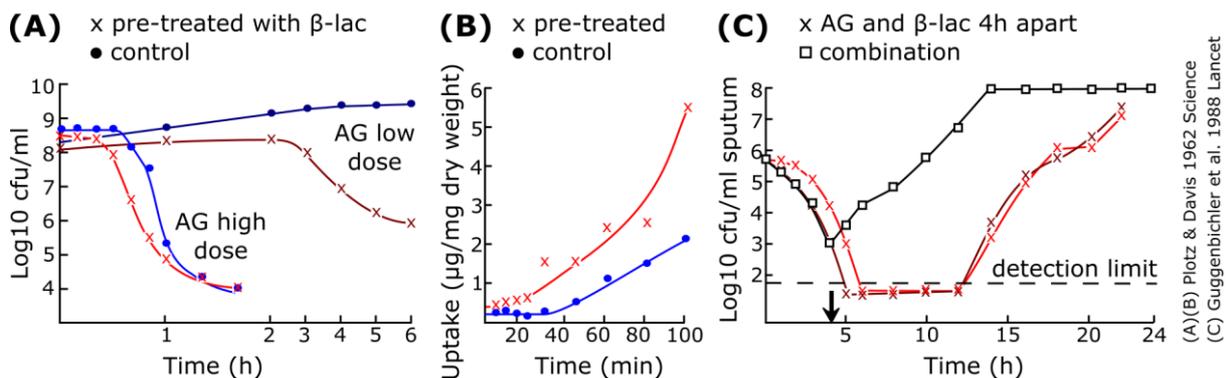
In this paper, we outlined how eco-evolutionary research can guide clinicians in the design of sustainable antibiotic treatments. We argue that current cycling treatments do not yet reach their full potential to eliminate bacteria and simultaneously minimize resistance evolution. Using eco-evolutionary principles, we identify fast sequential treatment of individual patients as a hard-to-adapt treatment option that warrants further exploration as a weapon against antibiotic resistances. By studying the emergence of resistance, evolutionary ecologists can contribute to the management of a growing global problem. Antibiotic resistance is an ancient ecological trait and its spread and rapid emergence are evolutionary processes. Clearly, evolutionary ecologists should get involved!

Box 1. The discovery of collateral sensitivity.

Collateral sensitivity is the specific term for trade-offs in antibiotic resistance, in which genetic changes that increase resistance to one antibiotic simultaneously increase susceptibility to other antibiotics. Collateral sensitivity was originally discovered and studied by Waclaw Szybalski at Cold Spring Harbor in the 1950s. Szybalski selected bacteria resistant to a wide array of antibiotics and toxic agents and screened them for cross-resistance against other antibiotics [80–82]. He discovered class-specific patterns in cross-resistance but also collateral sensitivity, and proposed to exploit these observations in chemotherapy [80]: *“Whenever one antibiotic can be found that is particularly effective against bacteria resistant to another, it might be proved useful in combating disease and in permitting the application of antibiotics in a rational sequence when more than one is to be employed. Thus, the exact study of both collateral sensitivity and cross resistance may help in designing a proper program of multiple chemotherapy.”* However, at the time, antibiotic resistance was not common and research did not follow up on his ideas. Instead, his findings were mainly applied in the search for novel antibiotics [83]. Candidate substances were used to select for resistant mutants, which were screened for their collateral sensitivity profiles. A deviation of the mutant profiles from established profiles was taken as indication of a new class of antibiotic. In the following years, the term collateral sensitivity disappeared from the field of antibiotics research, although studies continued to accumulate evidence of sensitivity trade-offs in antibiotic resistance [4,37,84,85]. Only now – in the light of the antibiotic crisis – has this concept been re-connected to antibiotic therapy [28], as originally proposed by Szybalski. Matrices of evolved collateral effects have now been inferred for *E. coli* and *P. aeruginosa* under laboratory, highlighting a high frequency of collateral sensitivities involving aminoglycosides, although their direction can vary among bacteria [28,29,31,86] and between evolved replicates of the same strain [31] depending on the precise genetic changes. The obtained insights are currently being explored for clinical application of collateral sensitivity in sequential treatment regimens.

Box 2. Sequential application potentiates treatment due to physiological interactions.

Short exposures to sub-lethal concentrations of antibiotic can potentiate subsequent antibiotic treatment. This phenomenon was first described in 1962 for *E. coli*. Pre-treatments of bacterial cultures with β -lactams for 15 minutes increased the bactericidal activity of aminoglycosides (AG, Fig. panel A, modified from [47]) by accelerating their cellular uptake (Figure panel B, modified from [47]). Such physiological effects are likely important in a clinical study on a cohort of cystic fibrosis (CF) patients with chronic *P. aeruginosa* lung infections, published in 1988 and representing one of the very few clinical applications of fast sequential therapy (i.e., including drug changes within a patient in less than a day). This study evaluated the potency of a specific form of sequential treatment, where a second antibiotic is added while the first antibiotic administered four hours earlier is still present in the patient at decreasing serum levels. Physiological interactions should influence treatment outcome, even if not known by the authors, because they switched between aminoglycosides and β -lactams, thus recapitulating the above described conditions. The test was unexpectedly successful, substantially reducing bacterial load upon sequential treatment (Fig. panel C, modified from [21]): “Between 1983 and 1987, 36 episodes of pseudomonas infections in thirty-two patients with CF have been treated with a combination of a β -lactam (azlocillin, piperacillin, ticarcillin 120 mg/kg) and an aminoglycoside (gentamicin or tobramycin 12 mg/kg) with doses 4 hours apart. In 16 episodes *P. aeruginosa* was eradicated from sputum for at least 3 weeks and sometimes for up to a year. In all other patients the number of colony forming units in sputum fell 1000-10000-fold. Clinical improvement, as judged by fever, amount of sputum, and laboratory findings (e.g. erythrocyte sedimentation) was seen in every patient.” [21] This strikingly contrasts with simultaneous dosing: “Between 1972 and 1978 we treated 66 episodes of infection due to *P. aeruginosa* in fifty-two patients with CF. We used a combination of carbenicillin (500 mg/kg) and an aminoglycoside (5 mg/kg) given simultaneously every 8h. In none of these 66 episodes was the pathogen eradicated.” [21] It is fascinating to see that this highly effective application of fast sequential therapy was not expanded and more widely explored.



Box 3. Outstanding questions.

With this paper we would like to stimulate research on evolution in complex drug environments. Many points warrant further exploration and some of these could be tackled with experimental evolution.

Main topic 1: Evolutionary questions

- What is the evolutionary robustness of adaptive constraints, such as collateral sensitivity or cross-stress sensitivity?
- How does evolution manage competing adaptive constraints? Specifically, when does evolution prioritize adaptation to physiological interactions or genetic trade-offs? How does phenotypic memory affect the emergence of resistance mutations?
- What is the mechanistic explanation for decreased fitness gains after stochastic compared to periodic selection?
- What is the optimal switching rate and treatment duration in sequential therapy? To what extent is the switching-rate optimum influenced by drug dose (i.e., selection coefficient), physiological interactions, or trade-offs?
- How does anticipatory gene regulation evolve? Although the parameter space is described, we lack an experimental demonstration for the evolution of anticipatory gene regulation.
- Which selective conditions favor the evolution of bet-hedging strategies such as phenotypic heterogeneity? Is this dependent on switching rate, stochasticity, or genetic trade-offs?

Main topic 2: Treatment-related questions

- What are the dynamics and likelihood for resistance emergence in multidrug-treatments with 3 or more antibiotics? Can the rate of adaptation be decreased by unpredictable order of antibiotics?
- How do bacteria evolve under sequential treatment which responds to evolution in real time? So far experiments assessed evolution in pre-defined scheduled protocols with balanced proportions of drugs. What happens when the replacement of antibiotics is directly coupled to real-time diagnostics by feedback loops?
- What are the molecular mechanisms for cross-stress sensitivity?

Highlights.

- Eco-evolutionary processes determine the rise in antibiotic resistance, yet are largely ignored in the current antibiotic crisis
- Eco-evolutionary principles can thus help the design of sustainable treatments, such as fast sequential therapy
- Fast sequential therapy creates temporal selective constraints that are difficult to adapt to, especially if combined with physiological effects, genetic trade-offs, or stochasticity
- Exploration of adaptation to fluctuating antibiotic environments should simultaneously enhance our understanding of the process of evolution, taking advantage of the comprehensive database on antibiotic resistance mechanisms and established experimental tools for pathogens
- Such work will further close the gap in our understanding of adaptation to stochastically fluctuating environments, which are widespread in nature but neglected in experimental studies

Glossary

Antagonistic pleiotropy: a mutation that influences several traits improves one trait and compromises other traits

Bet-hedging: constitutive or inducible expression of distinct phenotypes in isogenic populations as an adaptive strategy to persist in environments with unpredictable change

Clonal interference: competition between clones in asexual organisms, as determined by the complex of mutations contained in each of the clonal organisms, potentially leading to loss of advantageous mutations from the population

Collateral sensitivity: the phenomenon that a mutation conferring resistance to a specific antibiotic causes increased susceptibility to other antibiotics

Evolution experiment: experimental selection of a particular phenotype over many successive generations of an organism; derived and ancestral phenotypic states are usually compared using common-garden experiments

Fitness cost of resistance: reduction of the maximum growth rate achieved in drug-free environments of a resistant mutant compared to wild-type cells

Stochasticity: lack of predictable order

Trade-off: concept of traits being mutually restricted by a common resource or a common genetic mechanism

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Chapter 2

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Adaptive paths to escape collateral sensitivity cycling.

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Abstract

Evolution is at the core of the impending antibiotic crisis. Sustainable therapy must account for the adaptive potential of pathogens, *e.g.*, by exploiting genetic trade-offs of resistance mutations, which can produce hypersensitivity to other drugs (so-called collateral sensitivity). To date, the evolutionary stability and thus therapeutic applicability of reciprocal collateral-sensitivity remains unclear. Here we demonstrate experimentally that the model pathogen *Pseudomonas aeruginosa* cannot easily overcome collateral sensitivity, yet escape is occasionally possible via rare mutations. We further show that the application of the phenomenon can have three evolutionary outcomes: (i) population extinction, when bacteria fail to counter sensitivity to the second drug; (ii) maintenance of the double-bind, because gain of resistance causes reliable re-sensitization to previous drugs; or (iii) conversion of hypersensitivity into multidrug-resistance by the fixation of rare mutations. The prioritized adaptive path depends on drug order. Our identification of robust genetic trade-offs will contribute to novel antibiotic therapy.

Main text

Treatment of cancer and infectious diseases often fail because of the rapid evolution of drug resistance¹⁻³. Optimal therapy should thus anticipate emerging resistant variants and exploit their characteristics to improve treatment⁴⁻⁶. Thereby, the applied therapy may be one step ahead of evolution. In bacteria, and similarly in cancer^{7,8}, mutations that confer resistance to one drug can cause hypersensitivity to other drugs (i.e., collateral sensitivity)^{6,9-12}. Antibiotic cycling with drug pairs, for which this relation is reciprocal, has been proposed as a sustainable treatment strategy, because – in theory – it traps bacteria in a double bind^{6,13,14}. It is argued that inversion of selection causes serial re-sensitization during adaptation to either drugs, reminiscent of a flip-flop mechanism. The validity of this argument lacks a thorough experimental test, and may generally be questioned because evolution is not a deterministic process. Recent work with non-reciprocal collateral sensitivity¹⁵ indicates that re-sensitization is contingent on history, but the genetic mechanisms are unclear. Here we use two-step experimental evolution (Fig. 1a) and genomic analyses of the pathogen *Pseudomonas aeruginosa* to measure the evolutionary stability of

reciprocal collateral sensitivity. In particular, we previously evolved *P. aeruginosa* to high levels of resistance against several antibiotics¹¹ and identified two cases of strong reciprocal collateral sensitivity, between (i) carbenicillin (CAR) and gentamicin (GEN), and also (ii) piperacillin/tazobactam (PIT) and streptomycin (STR), which we here validated through dose response analyses (Fig. 1b, 1c). To assess whether or not switching drugs reliably selects for re-sensitization, we here challenged four clones from each of four resistant populations in a 12-day evolution experiment with increasing concentrations of the hypersensitive drug under the following four conditions: presence or absence of the antibiotic against which bacteria originally evolved resistance, and fast or slow increase of the second drug (Fig. 1a). Concentrations were increased using linear ramps, to facilitate evolutionary rescue (Supplementary Table 1). Our results thereby yield a conservative measure for the applicability of collateral sensitivity cycling.

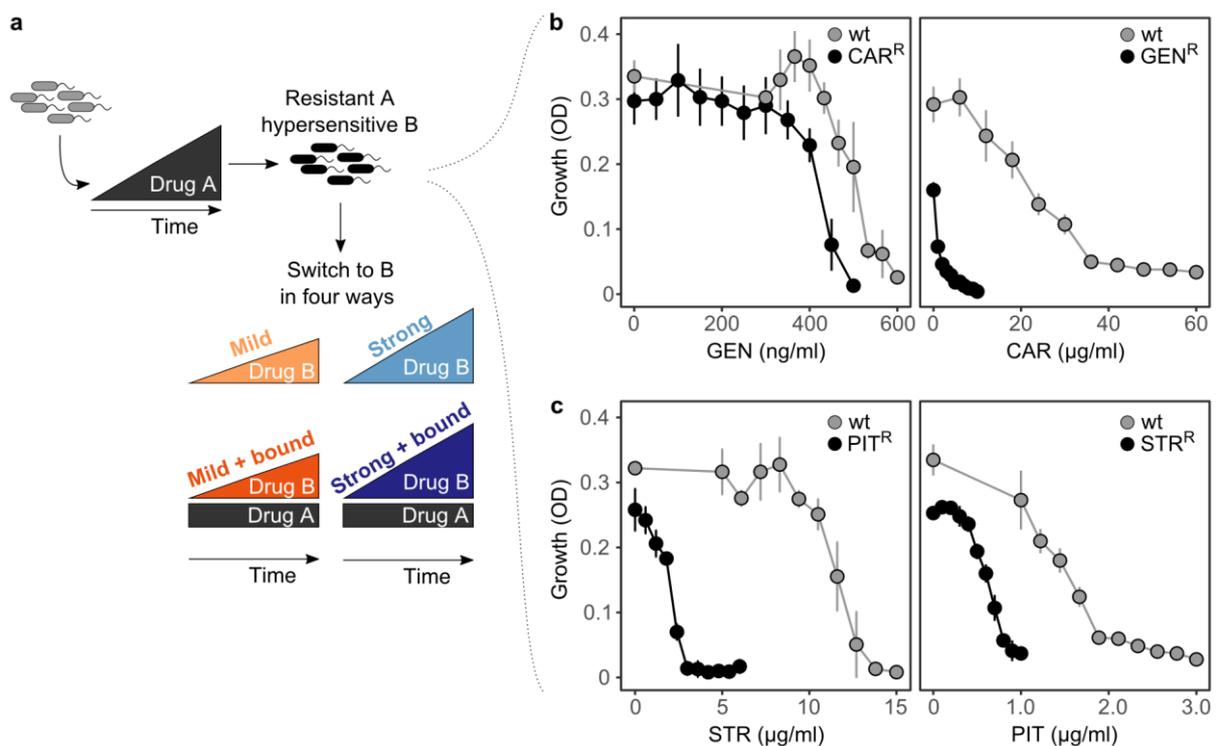


Figure 1. Reciprocal collateral sensitivity and experimental design. (a) Two-step experimental evolution: resistant populations of *P. aeruginosa* were experimentally selected with increasing concentrations of a particular drug (here labelled A) and resulting populations were hypersensitive to other drugs (here labelled B). In a second step, selection was inverted by switching treatment to drug B, with four selection regimes: (i) mild dose increase of drug B; (ii) strong dose increase B; (iii) strong dose increase B plus presence of drug A; and (iv) mild dose increase B plus presence A. Reciprocal collateral sensitivity for drug pairs (b) GEN/CAR and (c) STR/PIT. Mean \pm CI₉₅, 8 technical replicates. CAR, carbenicillin; GEN, gentamicin; STR, streptomycin; PIT, piperacillin with tazobactam; wt, wildtype; superscript R denotes resistance.

Evolutionary dynamics

Although, concentrations increased with small slopes (starting with IC₅₀ and ending at IC₉₅ of the hypersensitive or *wildtype* strains for fast and slow increases, respectively), experimental populations frequently went extinct, indicating strong genetic constraints for the evolution of

dual-resistance (Fig. 2). In particular, extinction was common when selection for the original resistance was maintained by the presence of both drugs (i.e., bound treatments), as compared to unbound evolution during sequential treatment (extinction events in bound vs. unbound treatments, χ^2 -test, $\chi^2=12.9$, $df=1$, $P<0.0001$; Fig. 2, Supplementary Table 2). During such unbound evolution, extinction only occurred under fast but not slow concentration increases. Throughout treatment, we monitored bacterial growth, using continuous absorbance measurements (optical density, OD_{600}). Relative biomass, as calculated from the areas under the obtained growth curves (AUC) relative to those from untreated controls, increased in surviving populations, indicating evolutionary adaptation. Adaptive increases in relative biomass were significantly slower for fast compared to slow increases, except for selection by STR (Figs. 2a, 2b, Supplementary Table 3). The simultaneous presence of both drugs significantly lowered growth across time, except for selection with PIT+STR (Fig. 2d; Supplementary Table 3). We conclude that adaptation to the second drug occurred in our experiments, although with varying degrees of difficulty. Adaptation was less rapid in the presence of both drugs, and for fast increases. These results are in agreement with previous studies that observed elevated extinction upon fast environmental deterioration as a consequence of narrowed mutation space^{16,17}. Interestingly, growth improvements appear to be facilitated when sequential treatments begin with the aminoglycosides rather than the β -lactam antibiotic of the pair (left panels versus the right panels in Fig. 2), possibly suggesting a drug order effect determining the dynamics of resistance evolution.

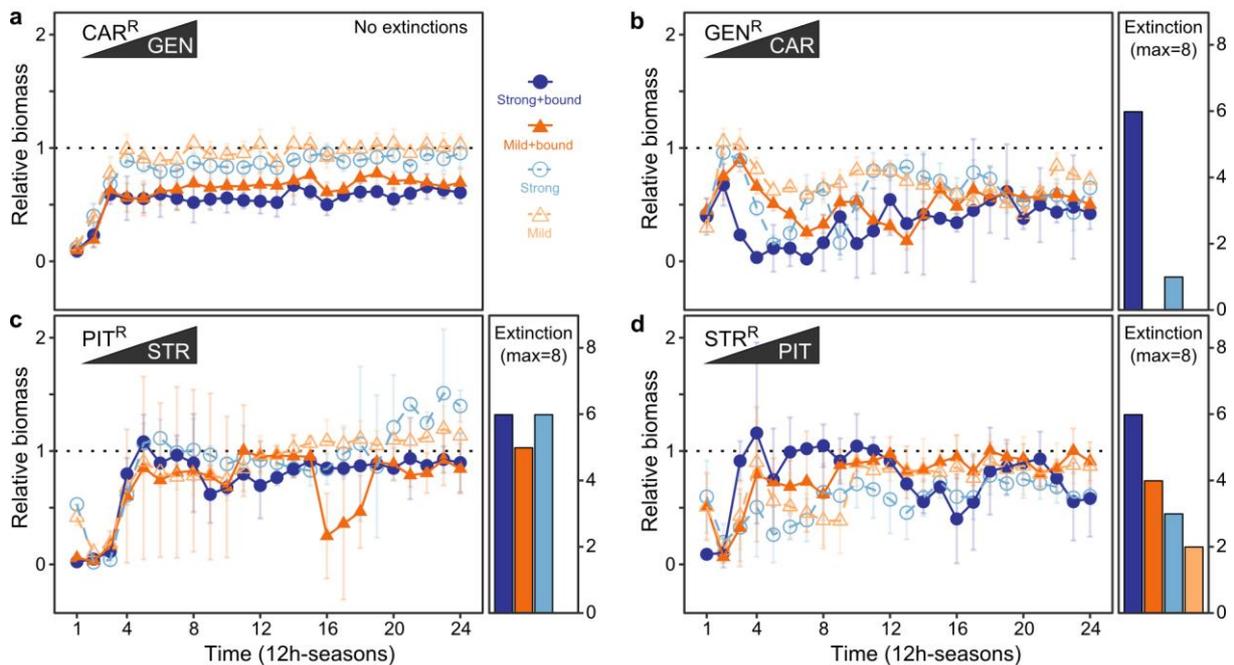


Figure 2. Growth dynamics and extinction events during second step of experimental evolution. Extinction events and changes in relative biomass of surviving populations for (a) CAR^R-populations adapting to GEN, (b) GEN^R-populations adapting to CAR, (c) PIT^R populations adapting to STR and (d) STR^R-populations adapting to PIT. The dotted horizontal line indicates growth equal to untreated controls. Mean \pm CI95, number of biological replicates differs due to extinction (Supplementary Table 2). Statistical evaluation of the differences among treatments is given in Supplementary Table 3.

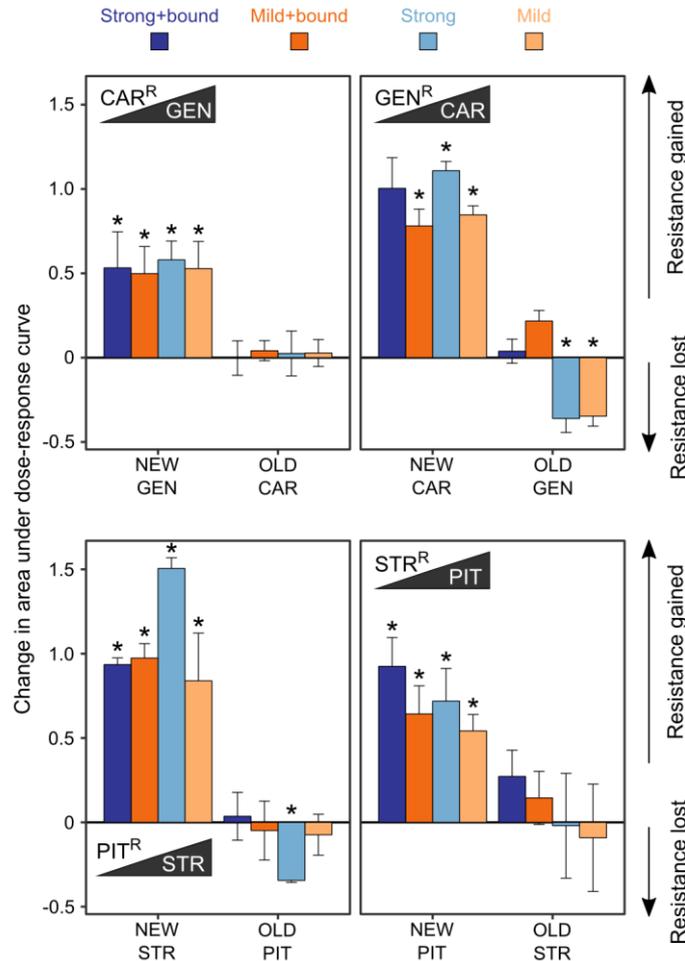


Figure 3. Changes in antibiotic resistance after one treatment cycle. Surviving populations from the various treatments (different panels and colors) were evaluated for changes in their resistance against the two antibiotics of a pair, which they experienced originally (OLD) or in the subsequent evolution experiment (NEW). The change is measured by cumulative differences in dose-response before and after the second round of evolution (i.e., the original antibiotic resistant clone *versus* its evolved descendants). Mean \pm CI95, number of biological replicates differs due to extinction (Supplementary Table 2). Asterisks indicate significant changes in resistance (one-sample *t*-test, $\mu=0$ per treatment and antibiotic; FDR-adjusted probabilities).

Evolution of re-sensitization

To evaluate whether selection by the second antibiotic re-sensitized bacteria to the original first drug or caused multi-drug resistance, we measured dose-response curves for the evolved populations against both antibiotics. In agreement with the recorded adaptive dynamics during treatment (Fig. 2), all surviving populations significantly increased resistance against the drug, towards which they originally produced hypersensitivity, regardless of whether both antibiotics were present or only one (Fig. 3; Supplementary Table 4). Resistance against the first drug always remained unchanged, when both antibiotics were present (Fig. 3). This demonstrates that the secondary adaptive mutations are not subject to the genetic trade-off originally responsible for collateral sensitivity, thereby highlighting that this particular evolutionary constraint can be overcome. Intriguingly, populations challenged with increasing concentrations of CAR or STR alone, showed significant decrease of resistance against GEN or PIT, thus restoring sensitivity

against these antibiotics. There was strong variation among lineages selected with PIT, i.e. only some lineages were re-sensitized to STR. Selection by GEN, did not re-sensitize against CAR. We conclude that *P. aeruginosa* has the genetic possibility to escape reciprocal collateral-sensitivity via rare cost-free resistance mutations. However, adaptation favors different mutational trajectories, where re-sensitization readily occurs, as witnessed with the antibiotics STR, CAR and PIT in the unbound treatments. The lack of re-sensitization upon selection with GEN indicates that the evolutionary stability of reciprocal collateral-sensitivity depends on the order of antibiotics during treatment.

Population genomics

To identify the genetic changes selected by treatment with the second antibiotic, we sequenced whole-genomes of the resistant starting clones and 35 evolved populations using samples from the end of the experiments. The samples for sequencing were selected thus: for each antibiotic at least all populations derived from one of the four starting clones and, additionally, when there were cases of re-sensitization in populations derived from other clones, all populations derived from those clones as well. Our analysis of the underlying genetic changes confirmed distinct evolutionary trajectories for bound and unbound evolution treatments, and explained the observed cases of re-sensitization (Supplementary Data 1).

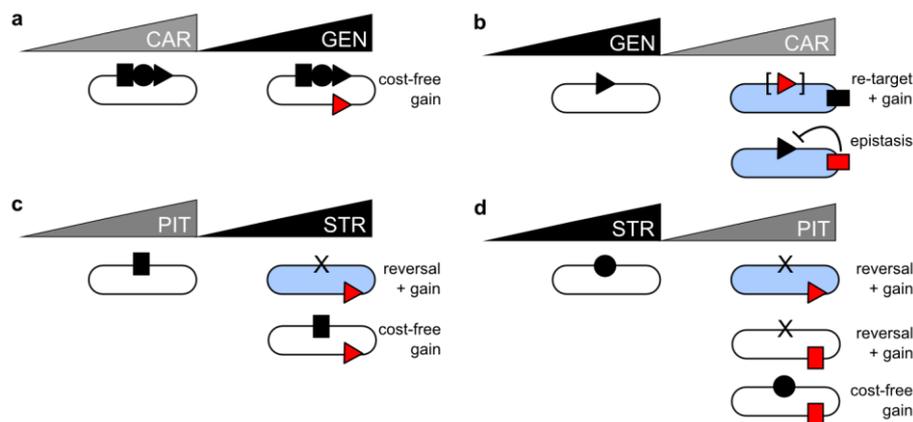


Figure 4. Genome dynamics during unbound treatments. (a-d) Different evolutionary trajectories during selection with the second antibiotic. Treatment order is illustrated with ramps. Shapes represent mutations in protein coding genes. Blue shading indicates re-sensitization to first antibiotic.

Genomics for the switch from CAR to GEN

The sequential selection with CAR followed by GEN resulted in a dual-resistant phenotype, which was explained by the sequential fixation of apparently cost-free resistance mutations (Fig. 4a). In detail, CAR^R was produced by a combination of mutations in (i) *nalC*, a TetR family repressor that controls expression of the multidrug efflux pump MexAB-OprM¹⁸; (ii) *ftsI/L*, which encode penicillin-binding-proteins¹⁹; and (iii) the two-component sensor *cpxS*, which likely contributes to envelope stress response²⁰. The mutation in *nalC* explains the collateral sensitivity to GEN¹¹. Subsequent gain of GEN^R was achieved by second-site mutations in the NADH-dehydrogenase genes *nuoD/G* (Supplementary Fig. 1), which are important for proton motive force such that mutations confer low-level resistance against aminoglycosides²¹. Alternatively, adaptation

occurred by mutation of *ptsP*, an important regulator for complex group behaviors associated to antibiotic resistance, such as biofilm formation²². Further mutational trajectories towards GEN^R involved changes in two-component sensors, as previously described^{11,23}. The diversity of mutational trajectories explained the observed rapid biomass increases during selection with GEN (Fig. 2a) and the lack of re-sensitization to CAR (Fig. 3). The genetic mechanism of *maintain and gain* was thus the first puzzle piece to explain the order-dependence in the adaptive responses during CAR/GEN cycling.

Genomics for the switch from GEN to CAR

When sequential treatments began with GEN followed by CAR, the emergence of CAR^R was associated with re-sensitization to GEN during unbound treatments (Fig. 3). The genomic analyses revealed two genetic mechanisms for the re-sensitization. The original resistance to GEN was caused by a non-synonymous substitution in the two-component sensor *pmrB* (Fig. 4c), which was also the molecular mechanism for hypersensitivity to CAR¹¹. Evolved re-sensitized bacteria had additional mutations in *nalC* that may increase CAR-efflux via *MexAB-OprM*¹⁸. Mutations in *nalC* were shown to mediate both resistance to CAR and hypersensitivity to GEN¹¹. Thus, re-sensitization to GEN may be caused by the antagonistic pleiotropy of *nalC* mutations that apparently override the still present pleiotropic *pmrB* mutation. A similar phenotypic shift was caused via a second regulatory pathway controlled by *nalD*²⁴, which is alternatively mutated in re-sensitized populations (Supplementary Fig. 2). A complementary mechanistic explanation for re-sensitization against GEN is re-mutation of *pmrB*. In three cases *nalC* mutations coincided with mutations in *pmrB*, including two deletions of 17 and 225 base pairs. Whilst the original SNP in *pmrB* alters gene function, the latter deletions may epistatically suppress the expression of the original SNP by pseudogenizing the gene (Fig. 4b). Altogether, unbound adaptation against CAR was achieved by mutations in the *nalC/D*-regulation of the *MexAB-OprM* pump, sometimes in combination with follow-up mutations in *pmrB*, and these mutations re-sensitized cells via epistasis.

Different mutational trajectories occurred when evolution of GEN^R was constrained by presence of CAR (Supplementary Fig. 1), explaining the observed differences in adaptive dynamics between bound and unbound evolution. High extinction frequencies indicated a greatly narrowed mutation space during bound evolution (Fig. 2b). Dual-resistance was achieved by the combined action of *mexR* and *phoQ*, an independent regulator of *MexAB-OprM*¹⁸ and a two-component regulator involved in aminoglycoside resistance²⁵, respectively. The evolution of a *mutS*-dependent hypermutator lineage (Supplementary Fig. 1) – an evolutionary strategy of last resort, because of the concomitant accumulation of deleterious mutations – highlights the comparatively high evolutionary stability of reciprocal collateral sensitivity when antibiotics are switched from GEN to CAR.

Genomics for the switch from PIT to STR

Cycling with the drug pair STR/PIT was associated with cases of re-sensitization regardless of drug order. Genetic analysis revealed that in both switching directions, ancestral resistance mutations were directly reverted to ancestral state (Fig. 4cd), indicating a lack of adaptive mutations and thus strong evolutionary stability of reciprocal collateral sensitivity for this drug pair. Resistance against PIT was mediated by *nalC* and *mpl*, a UDP-N-acetylmuramate: L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase involved in peptidoglycan synthesis²⁶, whereby the *nalC* variant most likely accounts for hypersensitivity to STR¹¹. Here, PIT^R populations adapted to STR by gain of mutations in *gidB*, which is known to contribute to STR resistance in *P.*

aeruginosa and other pathogens^{11,27} (Fig. 4c, Supplementary Fig. 3). *GidB* mutations only mildly increased resistance and stronger resistance required the mutational reversion of ancestral PIT^R-mutations, which occurred in several replicates. Gained STR^R is higher after mild STR increases than after strong or constrained ones, as indicated by the bar in the light blue in Fig. 3, due to reversal of *nalC* mutations in this treatment group. The mutational reversal also explained the observed re-sensitization to PIT (Fig. 3).

Genomics for the switch from STR to PIT

Cycling was likewise constrained in the opposite direction, where original STR^R was caused by a mutation in *gidB* (Supplementary Fig. 4). Interestingly, the mutation in *gidB* present in the starting clone was not found in $\frac{3}{4}$ of the sequenced populations, which had subsequently been challenged with PIT alone. Whether or not phenotypic re-sensitization occurred in these cases was contingent on the subsequently acquired resistance mutations. In spite of the mutational reversal, one of the populations showed dual-resistance, as explained by its subsequent fixation of a mutation in *mexR* that confers multidrug-resistance via over-expression of MexAB-OprM. In the other two cases, resistance against PIT likely occurred by mutations in *cpxS* and *PA14_41710*, and these populations were re-sensitized to STR. As expected, *gidB* mutations were maintained during simultaneous selection with both drugs. Further mutations in *nalC/D*, did not cause re-sensitization, indicating epistatic interactions between both genes that prevent the expression of their commonly associated collateral sensitivity against aminoglycosides¹¹. Overall, cycling between STR and PIT is generally stable, as reflected by frequent extinction and cases of re-sensitization. The observed variation between populations is caused by epistasis.

Conclusion

In summary, we experimentally tested the evolutionary stability of reciprocal collateral sensitivity in *P. aeruginosa*. While reciprocal collateral sensitivity between aminoglycosides and β -lactams generally limited resistance evolution in this pathogen, we observed that treatment outcome was contingent on drug order during sequential treatments. Treatments that started with aminoglycosides prevented the emergence of multi-drug resistance during subsequent β -lactam treatment. Our genomic analyses explain the underlying genetic mechanisms, namely phenotypic re-sensitization due to epistatic interactions between mutations or direct mutational reversal (Fig. 4). Conversely, sequential treatments that started with β -lactam and then switched to aminoglycoside were prone to evolutionary escape. Our results thus point to important limitations for the design of cycling treatments. High efficacy can be achieved by starting cycling treatments with the aminoglycoside (not the beta-lactam) and terminating therapy after two switches. We anticipate that our findings will contribute to the design of evolution-informed antibiotic therapy that controls infection and prevents the emergence of multidrug resistance.

Methods

Bacteria and media

All experiments were performed with the *Pseudomonas aeruginosa* PA14 lab strain and four derived resistant populations¹¹: *CAR-10*, *GEN-4*, *PIT-1* and *STR-2*. The resistant populations were previously selected for high level resistance against protein synthesis inhibitors from the aminoglycoside family; gentamicin (GEN; Carl Roth, Germany; Ref. HN09.1) or streptomycin (STR; Sigma-Aldrich, USA; Ref. S6501-5G), or alternatively cell-wall synthesis inhibitors from the β -lactam family; carbenicillin (CAR; Carl Roth, Germany; Ref. 6344.2) or piperacillin/tazobactam (PIT; Sigma-Aldrich, USA; Refs. P8396-1G and T2820-10MG). Tazobactam was supplied at the concentration of 1.0 μ g/ml. Antibiotic stocks were prepared according to manufacturer instructions and frozen in 100 μ l aliquots. Aliquots were kept frozen at -20°C for no more than 5 days, thawed only once and discarded after use. For isolation of clones, bacteria were grown on LB plates supplemented with the respective antibiotic at 37°C overnight. Evolution experiments and resistance measurements were performed in liquid M9 minimal media supplemented with glucose (2g/l), citrate (0.5g/l) and casamino acids (1g/l). These experiments were conducted in randomized 96-well plates, shaken (180rpm double orbital shaking) and incubated at 37°C in plate readers (BioTek Instruments, USA; Ref. EON), which recorded optical densities (OD₆₀₀) in 15 min intervals.

Genetic resistance trade-off

Previously, we identified reciprocal collateral sensitivity in evolved antibiotic resistant populations¹¹. Populations adapted to high concentrations of CAR and PIT had, respectively, increased sensitivity against GEN and STR, and *vice versa*. We confirmed the trade-off for this study by re-measuring the hypersensitivity of populations *CAR-10*, *GEN-4*, *PIT-1* and *STR-2* in comparison to wildtype PA14 (10 concentrations, 8 replicates). Cultures were grown to exponential phase, standardized by OD (OD₆₀₀=0.08) and diluted 10x into 96-well plates (total volume 100 μ l), yielding initial population sizes of $\sim 10^6$ CFU. Plates were shaken and incubated at 37°C for 12 hours. End-point measurements were then used to measure the dose-response relationship of each drug using the 'drc' package in the R platform²⁸.

Experimental evolution

To test the evolutionary stability of reciprocal collateral sensitivity, we challenged clones from previously evolved resistant populations with increasing concentrations of new antibiotics against which the resistant populations were hypersensitive: *CAR-10* with GEN, *GEN-4* with CAR, *PIT-1* with STR, and *STR-2* with PIT. Stability was assessed with 12-day evolution experiments with 2% serial transfers every 12 h. Each population was evaluated with 8 replicate populations (4 clones x 2 technical replicates) for each of 5 treatment groups: (i) untreated controls; linearly increasing concentration of hypersensitive antibiotic to a low level (ii) or high level (iii), without maintaining selection for previous resistance (unbound evolution); or linearly increasing concentration of hypersensitive antibiotic to a low level (iv) or high level (v), with simultaneous selection for previous resistance (bound evolution). Concentration increases were started with defined initial inhibition levels of IC₅₀ 50% of the starting clone (IC₅₀) and concluded when concentrations were above its IC₉₅ (mild increases) or IC₉₅ of the wildtype PA14 lab strain (strong increases), as specified in Supplementary Table 1. At the end of the experiment, evolved populations were frozen at -80°C in a 1:10 v/v proportion of sterile DMSO for characterization.

Dose-response measurements

We evaluated whether surviving lineages maintained or lost their ancestral resistance by exposing them to 2-fold concentrations of the drug they were challenged during experimental evolution and the one they were originally resistant to. Experiments were carried out as explained above, and included the starting clone of each evolved population as a control. We calculated the difference in resistance by subtracting the area under the dose-response curve of the evolved populations from that of the ancestral clones. Positive values indicate that the evolved lineages are more resistant than their ancestor, values close to zero suggest the resistance profile is equivalent and negative values highlight the loss of resistance.

DNA Extraction

We sequenced the genomes of 9 ancestral, and 35 evolved populations using samples from the end of the evolution experiments. The evolved populations were split thus: 9 from bound (3 strong+bound, 6 mild+bound), 16 from unbound evolution (7 strong, 9 mild) and 8 untreated controls. Frozen material was thawed and grown in 10ml of M9 minimal medium for 16-20h at 37°C with constant shaking. DNA was extracted using a modified CTAB buffer protocol²⁹. All DNA samples were sequenced at the Institute for Clinical Microbiology, Kiel University Hospital, using Illumina HiSeq paired-end technology³⁰ with an insert size of 150bp and 300x coverage. Overall, we found a total of 164 silent and 442 non-silent mutations (missense variants and short INDELS) in 63 protein-coding genes, including many previously characterized antibiotic resistance genes (Supplementary Data 1).

Genomic analysis

For the genomic analysis of *P. aeruginosa* PA14, we followed an established pipeline³¹. Briefly, reads were trimmed with Trimmomatic³², and quality-filtered with Skewer³³. We used the published *Pseudomonas aeruginosa* UCBPP_PA14_NC008463 genome available at (<http://pseudomonas.com/strain/download>) for mapping our samples. Mapping was performed using bwa and samtools^{34,35}, and manually inspected for low-quality areas using IGV (Integrated genome viewer, Broad Institute; www.broadinstitute.org/software/igv/). We used MarkDuplicates in Picardtools to remove duplicated regions for single nucleotide polymorphisms and structural variants (SNPs and SV). To call SNPs and small SV we employed both heuristic and frequentist methods, only for variants above a threshold frequency of 0.1 and base quality above 20, using respectively VarScan and SNVer³⁶. For larger SVs we employed Pindel and CNVnator^{37,37,38}. We used a combination of sources to annotate variants using snpEFF³⁹, DAVID, the *Pseudomonas* database (available online at: <http://pseudomonas.com>), and information from published work. Count statistics and data visualization were carried out in the R platform.

Acknowledgements

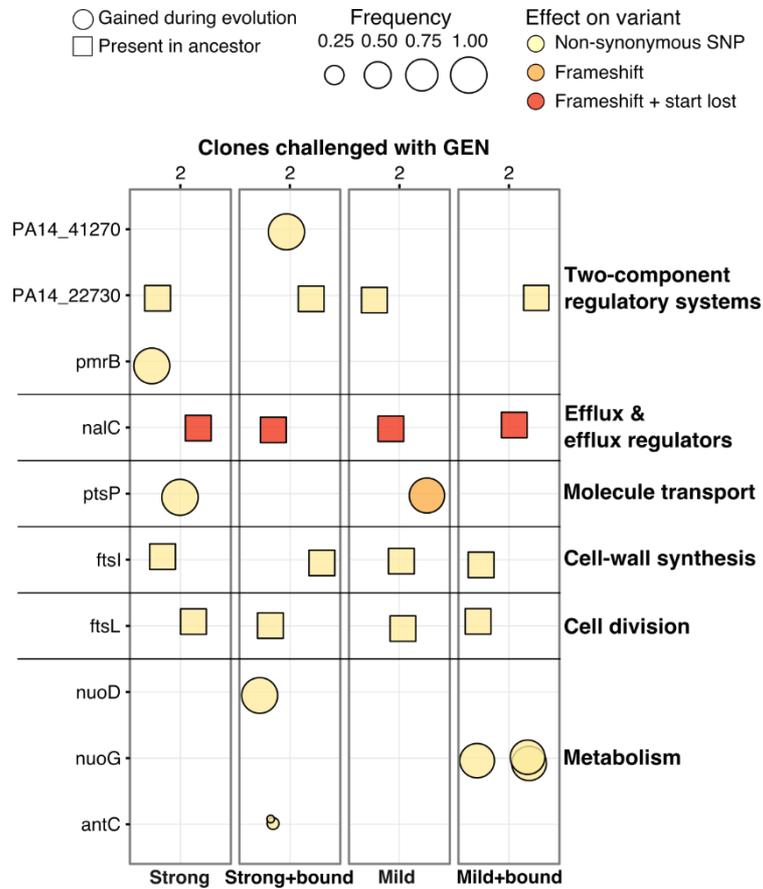
We thank the Schulenburg lab for helpful comments and advice; G. Hemmrich-Stanisak and M. Vollstedt from the Institute of Clinical Molecular Biology in Kiel for support with DNA sequencing, as supported by the DFG Cluster of Excellence “Inflammation at Interfaces”. We are grateful for financial support from the German Science Foundation (grant SCHU 1415/12 to H.S.), the International Max-Planck-Research School for Evolutionary Biology (C.B., R.R.), and the Max-Planck Society (H.S.). C.B., R.R. and H.S. designed experiments, C.B. and R.R. performed experiments, P.R. performed sequencing, all authors wrote the paper.

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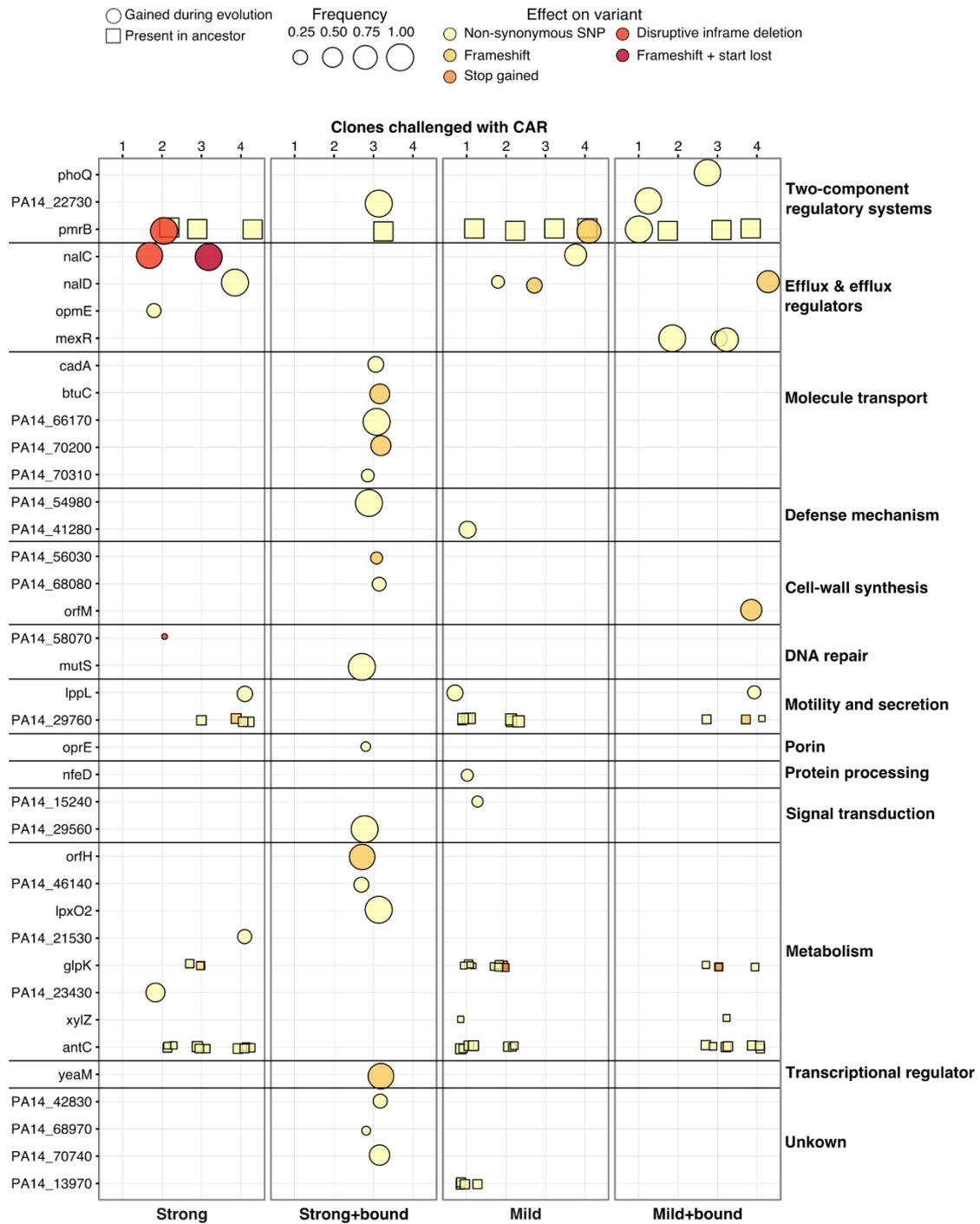
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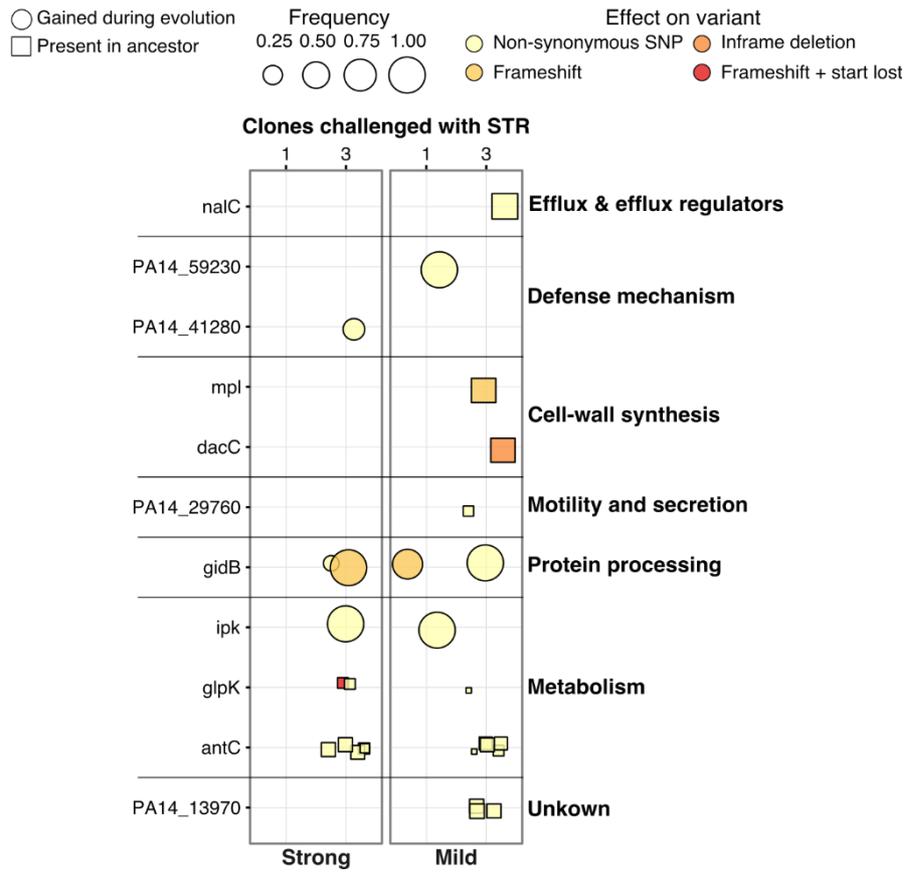
Supplementary material



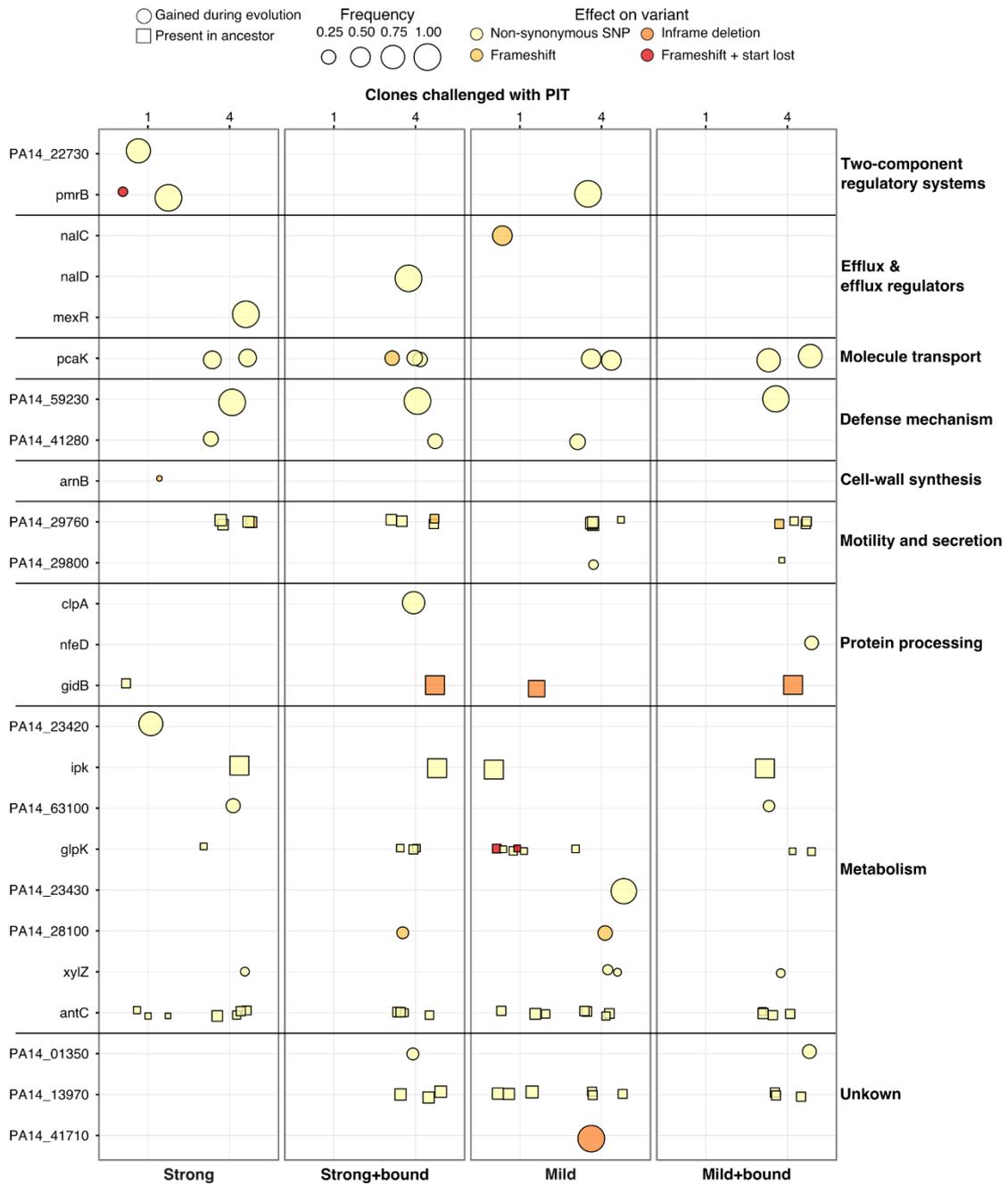
Supplementary Figure 1. Genomics of hypersensitivity reversal. Several populations started from distinct clones and challenged against GEN were selected for whole genome sequencing. Shown is the functional effect of mutations found in coding regions of the listed genes (vertical axis, left side) in the different starting clones (top horizontal axis), and across evolution experiments with different antibiotics (bottom horizontal axis). Functional information (right side) is inferred from a combined analysis using DAVID, the *Pseudomonas* database and publications. The shape of the point indicates whether it was found in the ancestor or only in the evolved population and the different colors highlight the effect of the variants found. The size of the points denotes the frequency at which the variant was found in the reads.



Supplementary Figure 2. Genomics of hypersensitivity reversal. Several populations started from distinct clones and challenged against CAR were selected for whole genome sequencing and analyzed as in Fig. S1.



Supplementary Figure 3. Genomics of hypersensitivity reversal. Several populations started from distinct clones and challenged against STR were selected for whole genome sequencing and analyzed as in Fig. S1.



Supplementary Figure 4. Genomics of hypersensitivity reversal. Several populations started from distinct clones and challenged against PIT were selected for whole genome sequencing and analyzed as in Fig. S1.

Supplementary Table 1. Antibiotic concentrations for evolution experiment.

Previously evolved resistant population	New antibiotic			For maintenance of original resistance*
	First dose (IC₅₀)	Final dose mild[†]	Final dose strong[§]	
CAR-10	410 ng/ml GEN	570 ng/ml GEN	890 ng/ml GEN	+87 µg/ml CAR
GEN-4	1.0 µg/ml CAR	30 µg/ml CAR	87 µg/ml CAR	+890 ng/ml GEN
PIT-1	2.2 µg/ml STR	8.5 µg/ml STR	21 µg/ml STR	+4 µg/ml PIT
STR-2	0.68 µg/ml PIT	1.8 µg/ml PIT	4 µg/ml PIT	+21 µg/ml STR

[†] IC₉₅ of hyper-sensitive population specified in column 1, [§] IC₉₅ of wildtype PA14, * added to treatment groups mild+bound, slow+bound.

Supplementary Table 2. Extinction events over time for each antibiotic used during experimental evolution and treatment (8 starting evolving populations).

Challenged with	Treatment (n=8)	Season		
		8	16	24
GEN	Strong	0	0	0
	Mild	0	0	0
	Strong & bound	0	0	0
	Mild & bound	0	0	0
CAR	Strong	1	1	1
	Mild	0	0	0
	Strong & bound	5	6	6
	Mild & bound	0	0	0
STR	Strong	5	6	6
	Mild	0	0	0
	Strong & bound	5	6	6
	Mild & bound	3	5	5
PIT	Strong	2	3	3
	Mild	2	2	2
	Strong & bound	6	6	6
	Mild & bound	3	4	4

Supplementary Table 3. Evaluation of the effect of the pace of drug increase (mild or strong) and evolutionary constraint (bound or unbound) on cumulative relative growth^a.

Antibiotic	Variable	χ^2	<i>P</i>	Adjusted <i>P</i>
GEN	Pace	14.7	<0.0001	0.0002
	Bound	158.1	<0.0001	<0.0001
CAR	Pace	18.1	<0.0001	<0.0001
	Bound	53.8	<0.0001	<0.0001
STR	Pace	2.3	0.1313	0.15
	Bound	29.4	<0.0001	<0.0001
PIT	Pace	9.6	0.0022	0.0023
	Bound	0.4	0.52	0.52

^a Separate GLMs were performed for each antibiotic used during experimental evolution with the cumulative relative growth of surviving populations as the response variable, and pace of drug concentration increase (strong or mild) and constraint (unbound or bound) as explanatory fixed factors. Starting clonal population was considered as a nested random factor. We used a type-II Wald χ^2 -test to evaluate the effect of these variables. We used the false discovery rate to adjust the *P* values for multiple comparisons.

Supplementary Table 4. Evaluation of the changes in resistance against two drugs after evolutionary reversal of antibiotic hypersensitivity*.

Resistant to	Challenged with	Treatment	Number populations	<i>P</i>	Adjusted <i>P</i>
CAR	CAR	No drug	8	0.92123	0.94485
		Strong	8	0.71558	0.7736
		Strong+bound	8	0.96151	0.96151
		Mild	8	0.514	0.6425
		Mild+bound	8	0.20661	0.29516
CAR	GEN	No drug	8	0.61526	0.72291
		Strong	8	2.00E-05	0.00013
		Strong+bound	8	0.00159	0.00489
		Mild	8	0.00031	0.00124
		Mild+bound	8	0.00044	0.0016
GEN	CAR	No drug	8	<0.00001	<0.00001
		Strong	7	<0.00001	<0.00001
		Strong+bound	2	0.05758	0.10237
		Mild	8	<0.00001	<0.00001
		Mild+bound	8	<0.00001	<0.00001
GEN	GEN	No drug	8	0.01936	0.03872
		Strong	7	0.00013	0.00065
		Strong+bound	2	0.47766	0.61634
		Mild	8	<0.00001	<0.00001
		Mild+bound	8	0.00023	0.00102
PIT	PIT	No drug	8	0.36843	0.49124
		Strong	2	0.01071	0.0252
		Strong+bound	2	0.70115	0.7736
		Mild	8	0.26425	0.36448
		Mild+bound	3	0.63255	0.72291
PIT	STR	No drug	8	0.04131	0.07869
		Strong	2	0.01344	0.0284
		Strong+bound	2	0.01349	0.0284
		Mild	8	0.00058	0.00193
		Mild+bound	3	0.00191	0.00546
STR	PIT	No drug	8	0.13735	0.21976
		Strong	4	0.00501	0.01252
		Strong+bound	2	0.05886	0.10237
		Mild	6	<0.00001	0.00057
		Mild+bound	4	0.00451	0.01203
STR	STR	No drug	8	0.0977	0.16283
		Strong	4	0.90519	0.94485
		Strong+bound	2	0.17739	0.2628
		Mild	6	0.58964	0.71472
		Mild+bound	4	0.1627	0.25031

* *P* values were obtained from a series of Student's t-tests per treatment for populations with ancestral resistance against a given antibiotic and evaluated against two drugs. We used the false discovery rate correction method to adjust *P* values for multiple comparisons.

Supplementary Data 1. Table of all mutated genes per population, antibiotic and treatment.

Sequenced population*	Treatment	Genetic variant ^s			Resensitization against first antibiotic	
		Freq.	Gene	Site		Length
CAR-10 (clone 2)	ancestor	1	ftsI	5116490	1	
CAR-10 (clone 2)	ancestor	1	ftsL	5118028	1	
CAR-10 (clone 2)	ancestor	1	nalC	1391016	-558	
CAR-10 (clone 2)	ancestor	1	cpxS	1977519	1	
b24_A3	mild	1	ftsI	5116490	1	-
	mild	1	ftsL	5118028	1	-
	mild	1	nalC	1391016	-558	-
	mild	1	cpxS	1977519	1	-
	mild	0.94	ptsP	393529	1	-
b24_F2	mild+bound	1	ftsI	5116490	1	-
	mild+bound	1	ftsL	5118028	1	-
	mild+bound	1	nalC	1391016	-558	-
	mild+bound	0.96	cpxS	1977519	1	-
	mild+bound	0.9	nuoG	2593381	1	-
	mild+bound	0.9	nuoG	2593382	1	-
	mild+bound	0.9	nuoG	2593383	1	-
b24_C1	strong	1	ftsI	5116490	1	-
	strong	1	ftsL	5118028	1	-
	strong	1	nalC	1391016	-558	-
	strong	1	cpxS	1977519	1	-
	strong	0.98	pmrB	5637059	1	-
	strong	1	ptsP	393833	1	-
b24_B2	strong+bound	1	ftsI	5116490	1	-
	strong+bound	1	ftsL	5118028	1	-
	strong+bound	1	nalC	1391016	-558	-
	strong+bound	1	cpxS	1977519	1	-
	strong+bound	0.98	nuoD	2596430	1	-
	strong+bound	1	parS	3683342	1	-
GEN-4 (clone 1)	ancestor	1	pmrB	5637090	1	
b24_H8	mild	1	pmrB	5637090	1	yes
	mild	0.35	PA14_41280	3685053	1	yes
b24_E9	mild+bound	1	pmrB	5637090	1	-
	mild+bound	0.95	cpxS	1977308	1	-
GEN-4 (clone 2)	ancestor	1	pmrB	5637090	1	
b24_A9	mild	1	pmrB	5637090	1	yes
	mild	0.31	PA14_29760	2577421	1	-
	mild	0.3	PA14_29760	2577418	1	-
	mild	0.26	PA14_29760	2577412	1	-
	mild	0.21	antC	2795876	1	-
	mild	0.2	nalD	1551588	1	-
b24_F8	mild+bound	0.99	pmrB	5637090	1	-
	mild+bound	0.99	mexR	486253	1	-
b24_C7	strong	1	pmrB	5637090	1	yes
	strong	1	pmrB	5637230	-225	yes
	strong	0.93	nalC	1390987	-5	yes
GEN-4 (clone 3)	ancestor	1	pmrB	5637090	1	
b24_D9	mild	1	pmrB	5637090	1	yes

	mild	0.29	nalD	1551588	-1	yes
b24_C8	mild+bound	1	pmrB	5637090	1	-
	mild+bound	0.74	mexR	486331	1	-
	mild+bound	0.95	phoQ	4369873	1	-
b24_F7	strong	1	pmrB	5637090	1	yes
	strong	1	nalC	1391237	-200	yes
b24_H7	strong+bound	1	pmrB	5637090	1	-
	strong+bound	1	lpxO2	4624709	1	-
	strong+bound	1	mutS	1501522	1	-
	strong+bound	0.88	orfH	2030085	1	-
	strong+bound	1	cpxS	1977213	1	-
	strong+bound	1	PA14_29560	2563755	1	-
	strong+bound	1	PA14_54980	4884493	1	-
	strong+bound	1	PA14_66170	5894687	1	-
	strong+bound	0.89	yeaM	4303970	1	-
	strong+bound	0.53	PA14_70740	6299422	1	-
	strong+bound	0.51	PA14_70200	6259755	-1	-
	strong+bound	0.5	btuC	2298608	1	-
GEN-4 (clone 4)	ancestor	1	pmrB	5637090	1	
b24_G7	mild	1	pmrB	5637090	1	yes
	mild	0.78	pmrB	5637232	-17	yes
	mild	0.62	nalC	1391004	1	yes
b24_A8	mild+bound	1	pmrB	5637090	1	-
	mild+bound	0.57	orfM	2039243	-8	-
	mild+bound	0.64	nalD	1551227	-8	-
b24_G8	strong	1	pmrB	5637090	1	yes
	strong	1	nalD	1551588	1	yes
PIT-1 (clone 1)	ancestor	0.8	mpl	1026509	1	
PIT-1 (clone 1)	ancestor	1	nalC	1391367	1	
b24_H5	mild	1	PA14_59230	5276901	1	yes
	mild	1	ipk	5510048	1	yes
	mild	0.66	gidB	6550306	-3	yes
PIT-1 (clone 3)	ancestor	0.87	mpl	1026509	1	
PIT-1 (clone 3)	ancestor	0.95	dacC	1046463	-3	
PIT-1 (clone 3)	ancestor	1	nalC	1391367	1	
b24_D6	mild	0.89	mpl	1026509	1	-
	mild	0.88	dacC	1046463	-3	-
	mild	1	nalC	1391367	1	-
	mild	1	gidB	6530122	1	-
b24_F4	strong	1	gidB	6550306	-3	yes
	strong	1	ipk	5510095	1	yes
STR-2 (clone 2)	ancestor	1	pcrD	3784765	1	
STR-2 (clone 2)	ancestor	0.97	ipk	5510095	1	
STR-2 (clone 2)	ancestor	0.75	gidB	6530306	-3	
b24_H11	mild	1	ipk	5510095	1	-
	mild	0.73	gidB	6530306	-3	-
	mild	0.5	nalC	1391216	-12	-
b24_E10	strong	1	pmrB	5637090	1	yes
	strong	0.8	cpxS	1977519	1	yes
	strong	0.78	PA14_23420	2035186	1	yes
STR-2 (clone 4)	ancestor	1	pcrD	3784765	1	

STR-2 (clone 4)	ancestor	1	ipk	5510048	1	
STR-2 (clone 4)	ancestor	0.75	gidB	6530306	-3	
b24_G10	mild	0.9	PA14_23430	2037005	1	yes
	mild	1	PA14_41710	3723476	-330	yes
	mild	1	pmrB	5637090	1	yes
	mild	0.49	pcaK	255917	1	yes
	mild	0.5	pcaK	255918	1	yes
b24_E12	mild+bound	1	ipk	5510048	1	-
	mild+bound	1	gidB	6530306	-3	-
	mild+bound	0.97	PA14_59230	5276901	1	-
	mild+bound	0.74	pcaK	255918	1	-
	mild+bound	0.75	pcaK	255917	1	-
b24_G11	strong	1	ipk	5510048	1	-
	strong	1	mexR	486350	1	-
	strong	1	PA14_59230	5276901	1	-
b24_F12	strong+bound	1	ipk	5510048	1	-
	strong+bound	1	gidB	6530306	-3	-
	strong+bound	1	nalD	1551486	1	-
	strong+bound	1	PA14_59230	5276901	1	-
	strong+bound	0.67	clpA	2618923	1	

* All samples from plate B, season 24; § Dark shading denotes ancestral mutations, light shading represents their maintenance in evolved populations. Freq., frequency.

Chapter 3

Manuscript, submitted to *Science*

Negative hysteresis improves antibiotic cycling efficacy.

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One-sentence summary

Antibiotics that induce physiological hysteresis reduce selection for drug resistance in sequential treatment protocols

Abstract

Antibiotic resistance can be mediated by inducible changes in cellular physiology. Surprisingly, such physiological effects are not part of the current concepts on the evolution of drug resistance. By combining experimental evolution, mathematical modelling, genomics, and functional genetics, we specifically tested whether bacterial evolution under sequential antibiotic therapy is shaped by negative hysteresis, which we here define as the survival-reducing physiological change induced by an earlier applied antibiotic. We demonstrate that sequential protocols with high frequencies of these physiological constraints impede resistance evolution, because selection favors an escape from negative hysteresis over resistance gains. Conversely, sequential protocols with little negative hysteresis enhance drug resistance. Our findings highlight the interplay between inducible physiological effects and resistance evolution and point to new ways of optimizing antibiotic therapy.

Main text

Natural environments are often temporally dynamic. They produce continuously changing selective constraints that are a particular challenge for organisms to adapt to (1). Similar dynamic conditions may be applied in our health system to limit the alarming ability of pathogens for resistance evolution. Antibiotic resistance is a global threat (2) and quickly growing by the emergence of new resistance mechanisms (3, 4). Cycling treatments may be one option to counter emerging resistance (5–8), for example when they exploit evolved collateral sensitivities, where the evolution of resistance against a first drug increases sensitivity to a second drug (9, 10). We here report a new approach for sequential drug treatments based on antibiotic-induced physiological constraints. Our approach takes advantage of hysteresis, which we here define as the change in physiology induced by a particular antibiotic that then alters susceptibility of an

individual cell to a second antibiotic, thus emphasizing the importance of prior experience for the physiological state of a particular cell (11). Negative hysteresis increases while positive hysteresis decreases susceptibility to the second drug, potentially reducing or enhancing bacterial survival upon drug change, respectively. The general phenomenon was already described in 1962 by Plotz and Davis in an attempt to explain the synergistic interaction between two simultaneously applied drugs. Short pre-treatments of *Escherichia coli* with penicillin caused transient damage to the cell wall that increased streptomycin uptake, leading to faster killing (12). Hysteresis has not yet been applied to drug cycling. Here, we specifically tested the potential of negative hysteresis to increase efficacy of cycling treatments using the human pathogen *Pseudomonas aeruginosa* as a model.

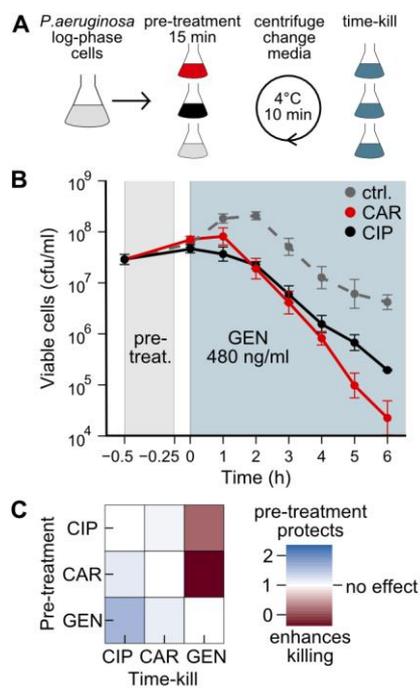


Fig. 1. Short antibiotic exposures affect killing by other antibiotics. (A) Schematic of time-kill experiment with 15 min pre-treatments. (B) Pre-treatments with non-lethal concentrations of ciprofloxacin or carbenicillin accelerate bactericidal activity of gentamicin, shown as concentration of viable cells (mean \pm SEM, 6 technical replicates). CAR, carbenicillin; CIP, ciprofloxacin; GEN, gentamicin. (C) Short pre-treatments can induce negative or positive hysteresis dependent on the direction of switches.

Hysteresis depends on the order of drug switches

We first characterized the hysteresis landscape (Fig. 1) of *P. aeruginosa* for three distinct and clinically relevant bactericidal antibiotics: the fluoroquinolone ciprofloxacin (CIP), the aminoglycoside gentamicin (GEN), and the beta-lactam carbenicillin (CAR) (13). As described for *E. coli*, we now found for *P. aeruginosa* that short pre-treatments with non-lethal concentrations of the beta-lactam increased killing by the aminoglycoside (i.e., negative hysteresis for CAR followed by GEN; Fig. 1B), while the reverse order slightly inhibited bactericidal activity (i.e., positive hysteresis; Fig. 1C; Fig. S1). We further identified a new hysteresis interaction between the aminoglycoside and the fluoroquinolone, GEN and CIP, two drugs with strong antagonistic interaction when simultaneously applied (Fig. S2). Pre-treatment with GEN caused positive, while the reverse direction negative hysteresis (Fig. 1C, Fig. S1). These results highlight that the sign of hysteresis can depend on drug order and that hysteresis and drug interaction are not necessarily linked, as originally assumed by Plotz and Davis (12).

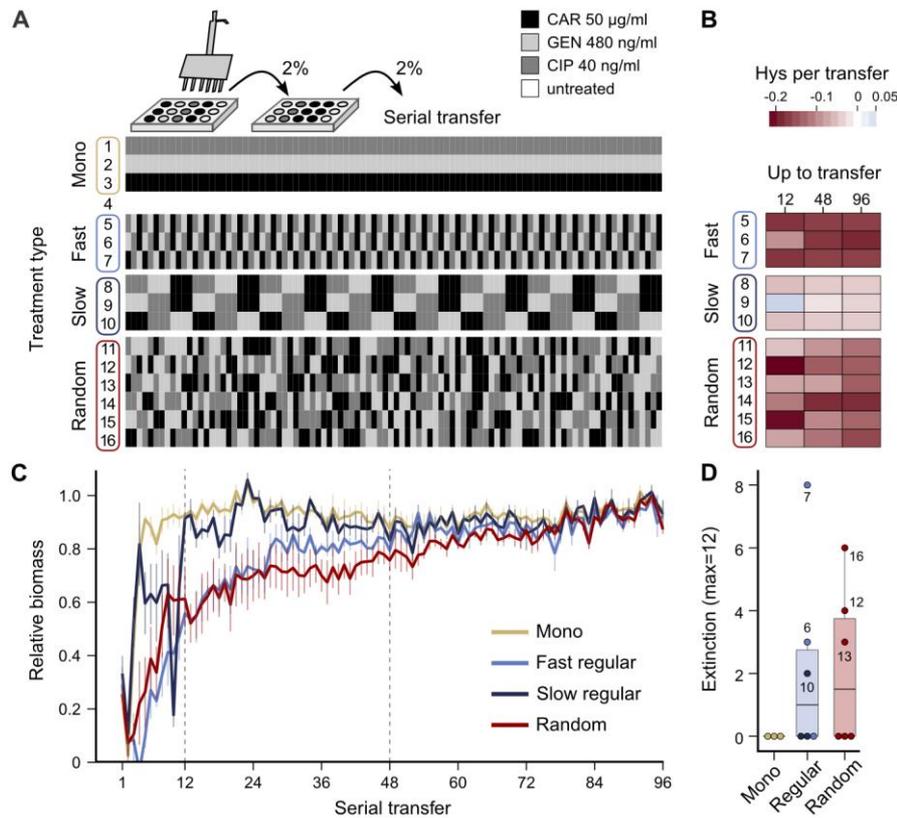


Fig. 2. Fast antibiotic cycling can constrain resistance evolution. (A) Schematic of evolution experiment with 16 different sequences. (B) The level of negative hysteresis differs between and within treatment types. Hys per transfer, cumulative hysteresis factor along treatment divided by number of transfers. (C) Evolutionary dynamics, expressed as total growth relative to evolving untreated controls (mean \pm CI95; 3-6 sequences per treatment type and 12 biological replicates per sequence; extinct lineages excluded). (D) Variation of extinction frequencies per treatment. Numbers indicate sequence as in (A).

Experimental evolution reveals constrained adaptation under fast sequential protocols

To assess the potential of hysteresis for cycling therapy, we conducted a high-throughput evolution experiment with 190 replicate populations over a total of 96 transfers (~500 generations). We included three main types of cycling protocols, in order to disentangle the influence of hysteresis from the frequency and also temporal regularity of drug switches. Two main types were regular but at different switching rates (*e.g.*, fast vs. slow switches; Fig. 2A), while the third main type consisted of random drug orders (Fig. 2A). Within each type, several distinct sequences were included that varied in hysteresis level (Fig. 2B) and starting drug, while the overall proportion of the three antibiotics was equal. Across the 96 transfers, the observed evolutionary dynamics consisted of three main phases: an initial increase in growth yield within the first 12 transfers, then a phase of gradual improvement until approximately transfer 48, and thereafter a phase with little change (Fig. 2C). During the first two phases, fast-regular and random sequences led to significantly smaller biomass increases in comparison to the monotherapies, whereas slow regular sequences reached almost the high control biomass levels already in the second phase (Fig. 2C; statistics sheet 1; figs. S3-6). Some replicate populations went extinct and did so significantly more often in the fast-regular protocols (Fig. 2D; statistics sheet 2). Extinction was also elevated in some random protocols. We conclude that the two treatment

types with fast drug changes constrained evolutionary adaptation and increased extinction frequencies.

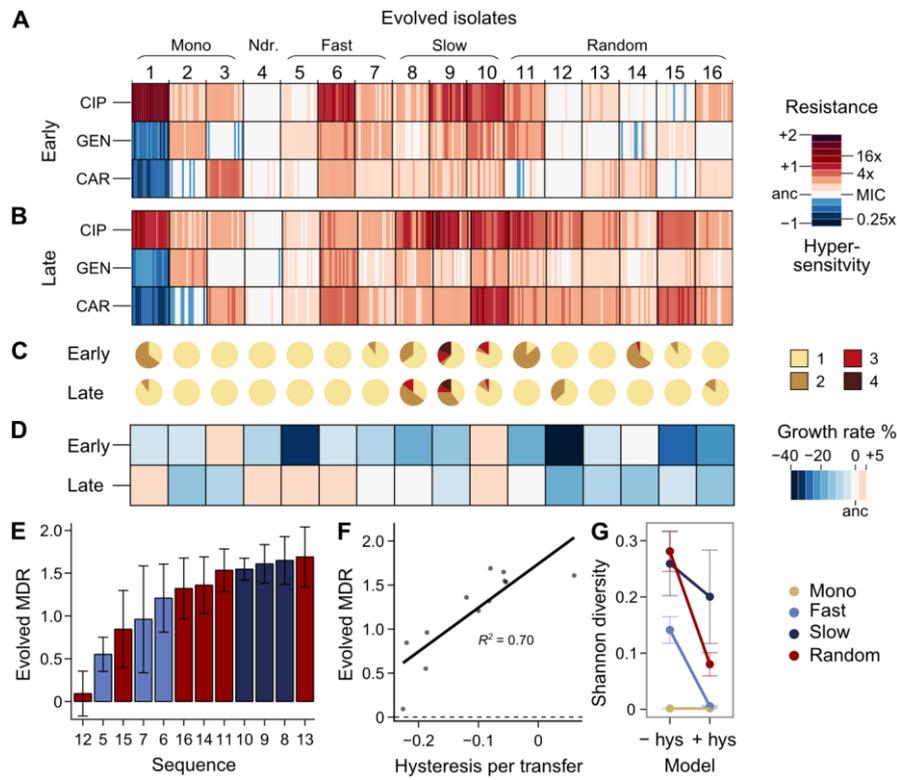


Fig. 3. Fast sequential treatments constrain early resistance evolution, due to hysteresis and evolved tolerance. (A) Resistance profiles of 320 clones isolated after transfer 12 from 16 populations; the clones are indicated by bars within the boxes for a particular treatment. (B) Resistance profiles of 320 clones isolated from the same populations after transfer 48. (C) Within-population diversity as determined by hierarchical clustering of resistance profiles at the same early and late time points. Different colors denote the distinct types per population. (D) Growth rate under drug-free conditions for the same two time points. Low growth combined with no resistance indicates tolerance (see #12 early time point). (E) Multidrug resistance (MDR) across sequences, inferred from an expansion of the initial analysis with a total of 880 isolates (mean \pm SEM, $n = 3$ -6 populations, 5-20 clones per population). (F) The experienced degree of negative hysteresis is significantly correlated with MDR levels after transfer 12. (G) A mathematical model tailored to our experimental design predicts that fast cycling leads to reduced population diversity in the presence (+hys) but not absence (-hys) of hysteresis.

Hysteresis reduces population diversity in fast cycling

We next assessed in more detail the evolved changes by characterizing 20 bacterial isolates of one representative population from each of the 16 treatments at two time points, defining the approximate end of the early (transfer 12) and the middle phases (transfer 48). For the resulting 640 isolates, we determined antibiotic resistance profiles and growth under drug-free conditions. In agreement with the recorded evolutionary dynamics (Fig. 2C), mean resistance varied substantially among sequences at the early time point (Fig. 3A), and less so at the later time point, at which resistance had generally increased (Fig. 3B). Surprisingly, we found significantly fewer resistance types (13) in populations from fast than slow-regular sequences (Fig. 3C; figs. S7, S8; statistics sheet 3). Populations from fast-regular sequences were mostly dominated by a single

type, whereas populations from slow-regular sequences were divided into 3-4 types. The presence of such sub-populations was validated with a complete repetition of the measurements (14) (Fig. S9; statistics sheet 4). Moreover, whole-genome sequencing of a representative subset of the isolates confirmed that the sub-populations were genetically distinct (Table S1). These results contrast with expectations from population genetic theory, because fast switching should have rather prevented competitive exclusion and, instead, caused oscillation of multiple types parallel to antibiotic exposure. To assess these dynamics in more detail, we developed and analyzed a mathematical model tailored to the design of the evolution experiment (13). Under standard conditions, the model captured the expected clonal interference and coexistence of several types under fast cycling conditions (Fig. 3G). Importantly, when we added hysteresis effects to the model, then we found increased selection pressure and a reduction of diversity, especially for the fast treatments (Fig. 3G; figs. S10-S13). These observations suggest that hysteresis acts as a strong selective constraint during drug cycling and influences diversity within the evolving populations.

Hysteresis is a main determinant of evolved multidrug resistance

We next asked whether differences in hysteresis levels are associated with variation in evolved drug resistance and other phenotypic traits. Resistance varied among and also within the main treatment types, most strongly at the early time point (Fig. 3A). Intriguingly, populations treated with sequence #12 had zero resistance at transfer 12 (Fig. 3A), and similar success was achieved by sequences #5 and #15, in which resistance only increased marginally. To validate the variation of drug resistance, we used a larger sample of isolates (240 additional isolates across replicates) to calculate a score for multidrug resistance, MDR (13). MDR was significantly lower after fast-cycling than after slow-cycling (statistics sheet 5), yet the best and the worst cycling treatments were random sequences (Fig. 3E), suggesting that next to treatment type, the exact drug order and thus possibly the cumulative level of negative hysteresis is crucial. The highest cumulative level of negative hysteresis is achieved by sequence 12 (Fig. 2B), in which mean MDR was not significantly different from zero (measurements based on all surviving populations; statistics sheet 6). Indeed, hysteresis levels were significantly correlated to evolved MDR (Fig. 3F; statistics sheet 7), with the lowest resistance corresponding to the highest measure of negative hysteresis. Evolved MDR was also negatively associated to switching rate, but to lesser degree (Fig. S14). Moreover, hysteresis levels but not switching rates were significantly correlated with rates of biomass increase (Fig. S14). We conclude that even though switching rate is important, the consideration of hysteresis is sufficient to predict treatment efficacy under our experimental conditions, and that treatment efficacy is maximized by the abundance of negative hysteresis.

Interestingly, the mathematical model indicated that negative hysteresis increases selection intensity (Fig. S12), yet the observed outcome was not MDR – as would be expected from competitive release (15) – but rather a constrained ability to evolve MDR (Fig. 3F). Thus, we hypothesized that hysteresis diverts adaptation towards unusual adaptive peaks. The presence of an alternative evolutionary response specific against negative hysteresis is indicated by our additional analyses of growth rate (13). Almost all drug protocols resulted in reduced growth rates under drug-free conditions (Fig. 4D, validated with colony counts, Table S2; statistics sheet 8), but the three sequences (#5, #12, #15) with high negative hysteresis and almost no evolved MDR showed the strongest growth reductions of up to 42% (Fig. 3D). The combination of reduced growth under drug-free conditions and high drug susceptibility is indicative of antibiotic tolerance (16), which could thus have been favored through selection by negative hysteresis.

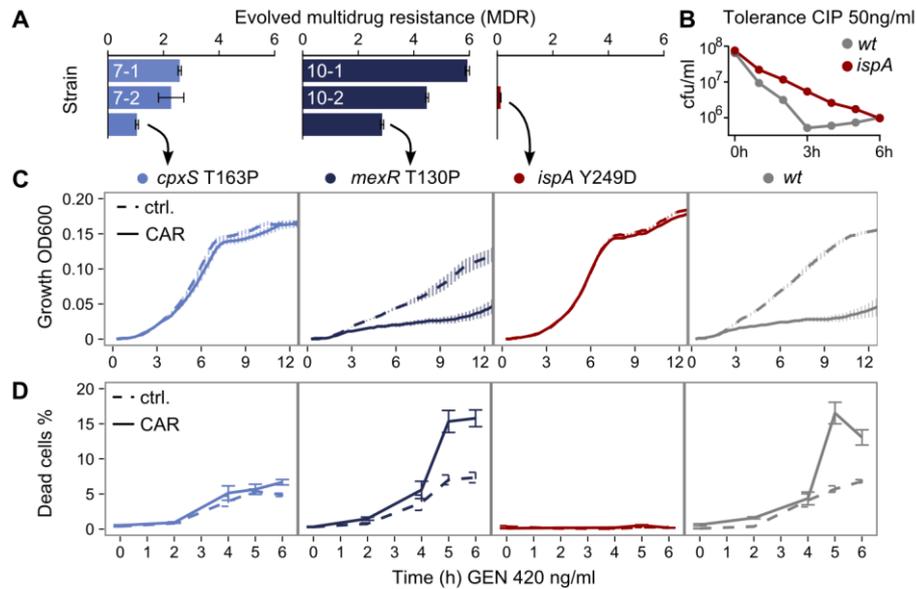


Fig. 4. Evolutionary adaptation to negative hysteresis. (A) Evolved multidrug resistance of isolates from fast, slow and random cycling and constructed mutants with corresponding mutations (mean \pm SEM, 6-20 technical replicates). The top two bars refer to the isolates, the bottom bars to the defined mutant. (B) Isolate from #12 with mutation in *ispA* shows antibiotic tolerance and thus reduced cellular death over time. (C) Presence of negative hysteresis in different genotypes. Pre-treatments with CAR (solid lines) inhibited subsequent growth in the presence of GEN in *wt* and *mexR* T130P, but neither in *cpxS* T163P nor *ispA* Y249D mutants (mean \pm SEM; 6 technical replicates). (D) Confirmation of hysteresis phenotypes as the dynamics of dead cells over time by flow-cytometry (mean \pm SEM; 3 technical replicates).

Negative hysteresis favors genetic changes mediating tolerance and a novel response

To further validate the selective impact of negative hysteresis during sequential therapy, we characterized the genes that have likely been the targets of selection using whole-genome sequencing of 30 phenotyped isolates (Fig. 3), followed by functional genetic analysis in selected cases (13). The genomic characterization revealed different sets of mutations to be favored by the main treatment types (Table S1; figs. S15, S16). Isolates from the most effective protocols with highest cumulative levels of hysteresis (sequences #5, #12, #15) did not have any unique mutations in common (Table S1). Yet, the sequenced isolate from protocol #12 harbored a mutation that is likely to mediate tolerance. Based on time-kill experiments (17) with this isolate, we could indeed confirm antibiotic tolerance, including absence of resistance, decreased growth in the absence of drugs combined with reduced killing rates on all three antibiotics relative to the ancestor (Fig. 4B; Tables S2, S3). The isolate has two mutations, one leading to an amino acid change in *ispA* (Y249D) and a frame shift in the glycine cleavage gene *gcvT2*. Because the *gcvT2* mutation occurred across treatment groups (Table S1), we propose that the *ispA* mutation is the adaptive mutation that caused tolerance through reduced growth, most likely due to the toxic accumulation of isoprenyl diphosphates, as previously recorded for a Δ *ispA* *E. coli* deletion mutant (18). In the first stage of the evolution experiment, sequence #12 was enriched for the negative hysteresis switch CAR- \rightarrow GEN. A re-assessment of the CAR- \rightarrow GEN hysteresis showed that negative hysteresis could no longer be induced in this isolate (figs. 4C, 4D). We conclude that selection by negative hysteresis in sequence #12 was countered by the emergence of antibiotic tolerance, mediated through a mutation in *ispA*.

One of the other effective protocols, #5, had a mutation in the previously uncharacterized, putative two-component sensor *cpxS* (*PA14_22730*, Fig. S14), which is related to the *E. coli* envelope stress response system CpxA-CpxR. This stress response system is activated by misfolded proteins, as caused by aminoglycosides (19), and involved in intrinsic resistance to these drugs in *E. coli* (20). *CpxS* mutations were significantly enriched in fast-regular protocols (statistics sheet 9), including those with little indication of antibiotic tolerance. Even though it may not cause tolerance, this gene could still contribute to the evolutionary response against negative hysteresis. To explore this idea, we re-introduced one prevalent *cpxS* mutation (leading to the amino acid substitution T163P) into the ancestral background (13) and compared it to the ancestor and also the similarly generated *mexR* mutant as a control (including a T130P amino acid substitution found in an isolate from slow regular sequence #10). MexR regulates the multidrug efflux pump MexAB-OprM, which can extrude the three drug classes (21), potentially conferring complete resistance. The tested *mexR* mutation is unlikely favored by negative hysteresis as this gene was only mutated under slow-regular conditions (Fig. S15; statistics sheet 9). Our analysis now revealed that resistance against CIP and CAR was moderate for the *cpxS* mutant, but strongly increased for the *mexR* mutant, while neither mutation altered resistance to GEN (figs. 4A, S17). Importantly, using two independent methods to assess CAR->GEN hysteresis (13), we consistently found that negative hysteresis was abolished in the *cpxS* mutant, but still present in the *mexR* mutant (figs. 4C, 4D). We conclude that mutations in *cpxS* were most likely favored in fast cycling protocols to counter negative hysteresis, independent of antibiotic tolerance, while *mexR* mutations enhanced antibiotic resistance in slow-regular sequences.

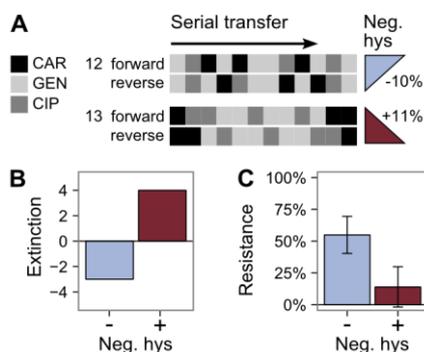


Fig. 5. Reversal of sequences predictably alters treatment efficacy due to changes in hysteresis. (A) Reversal of the first 12 treatment steps in two sequences changes hysteresis characteristics. Neg. hys, cumulative negative hysteresis. (B) Change in hysteresis predictably changes extinction frequencies. (C) Decreasing hysteresis significantly increases resistance in surviving lineages (mean \pm SEM, $n = 3$ antibiotics).

An independent experimental test validates the importance of negative hysteresis

Inspired by Lewontin (22), we specifically re-evaluated the influence of hysteresis by repeating evolution experiments with the reversed order of drugs for the most effective sequence #12 and the least effective sequence #13. The reverse sequences had the same drug proportions and the same number of switches as the original sequences (Fig. 5A), but the direction of the transitions was opposite. As a consequence, all was equal except that the cumulative level of negative hysteresis was decreased by 10% in the first case and increased by 11% in the second case. As expected, reversing #12 decreased extinction frequency (Fig. 5B) and significantly increased resistance gains (Fig. 5C; statistics sheet 10). Conversely, reversing #13 increased extinction (Fig. 5B), although resistance was not affected (statistics sheet 10), most likely because only few populations survived and could thus be used for resistance analysis. These results clearly demonstrate that negative hysteresis can determine the efficacy of fast sequential therapy.

Conclusions

We here report that antibiotics can induce changes in bacterial cellular physiology that increase or inhibit the bactericidal activity of other antibiotics and can be exploited by sequential therapy to maximize treatment efficacy. Fast changes between antibiotics are key, because they can increase the cumulative effect of negative hysteresis, leading in our experiments to a reduction in population phenotypic variation, the rate of biomass increase and MDR. We confirm that selection by negative hysteresis does not favor resistance mutations but rather mutations that counter the inducible physiological effects, such as those here demonstrated for *ispA* and *cpxS*. In contrast, we also show that slow drug changes enhance resistance evolution and thus target different sets of genes. Our findings may explain the limited success of antibiotic cycling in the clinic, where antibiotics are usually changed once per month or less often (23). They may also explain the results from one of the few clinical tests of fast cycling, published in 1988 and so far widely ignored, which demonstrated that the staggered application of drugs four-hours apart causes a significant reduction and often full clearance of *P. aeruginosa* from the lungs of a small cohort of cystic fibrosis patients (24). A further exploration of negative hysteresis may help to find new ways for improving antibiotic therapy – with the available drugs and thus without the need to isolate and characterize new antibiotic substances.

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List of supplementary materials

Materials and Methods

Fig S1 – S19

Table S1 – S3

References (25 – 44)

Statistics excel file with separate sheets for the tests

Negative hysteresis improves antibiotic cycling efficacy.

Materials and methods

Experimental design

The objective of this work was to investigate how physiological constraints affect the evolution of pathogens during antibiotic sequential therapy. To address this question, we performed parallel evolution experiments with different sequential treatments.

Design of antibiotic sequences

We manually generated three regular sequences with three distinct antibiotics, ciprofloxacin (CIP), gentamicin (GEN), and carbenicillin (CAR). Each sequence started with a different antibiotic. The regular treatments were carried out at two switching frequencies, yielding treatments ##5 -7 with switches every 12h and ##8-10 with switches every 48h. We further generated six cycling treatments ##11-16 with random order from atmospheric noise (random.org, Randomness and Integrity Services Ltd., Ireland; Random Sequence Generator, timestamp 2015-05-31 10:07:29 UTC) and rotated them to obtain two treatments each starting either with CIP, GEN or CAR. All sequential treatments contained equal frequencies of CIP, GEN and CAR, but differed in their levels of negative hysteresis. Sequences are shown in Fig. 2A and the variation in hysteresis in Fig. 2B. Overall, this experimental design allowed us to disentangle the influence of hysteresis from starting drug, cycling rate, and switching regularity, which are all likely to influence evolutionary adaptation of bacteria to antibiotic sequential therapy.

Strains and media

All experiments were started with *Pseudomonas aeruginosa* UCBPP-PA14 (abbreviated 'PA14' or 'wt' for wildtype), a pathogen with broad host-range that was originally isolated from a human burn wound (25). We grew bacteria in M9-minimal media supplemented with glucose (2g/l), citrate (0.5g/l) and casamino acids (1g/l), to which we added antibiotics where required.

Carbenicillin (CAR; Carl Roth, Germany; Ref. 6344.2) is a beta-lactam antibiotic that inhibits transpeptidase-activity during cell wall synthesis. The fluoroquinolone antibiotic ciprofloxacin (CIP; Sigma-Aldrich, USA; Ref. 17850-5G-F) inhibits the unwinding of DNA during DNA replication. Gentamicin (GEN; Carl Roth, Germany; Ref. HN09.1) is an aminoglycoside antibiotic that inhibits translation of protein synthesis but also produces membrane damage. These antibiotics are commonly used for clinical treatment of *P. aeruginosa*. The antibiotics were dissolved, and stored according to manufacturer's recommendations in aliquots for single use. Fresh stocks were prepared in fixed intervals.

Time-kill experiments and hysteresis measurements

To measure hysteresis, we performed time-kill experiments with short pre-treatments as previously described (12). Bacteria were grown to exponential phase ($OD_{600} = 0.08$, 8×10^7 colony forming units (cfu)/ml) and the culture was split into 10 ml cultures and pre-treated with antibiotics for 15 min, or not treated. It is important, that these pre-treatments were non-lethal, which we confirmed with cfu-counts and by live-dead staining and flow cytometry. Thereafter, cells were pelleted by centrifugation (10 min, 4000rpm, 4°C), media discarded and pellets re-suspended in media containing an antibiotic and incubated at 37°C and orbital shaking. Samples were taken in 1h intervals for a total of 6h and plated on LA plates in serial dilutions. Cfus were counted after 24h incubation at 37°C. Concentrations were CIP 40 ng/ml, GEN 480 ng/ml, CAR 50 µg/ml. Time-kill data is shown in figs. 1B, S1 and S11.

Hysteresis was quantified by subtracting cfu-counts of pre-treated and untreated cultures. Cfucounts were corrected for the growth of the untreated cultures during the 15min pre-treatment. Hysteresis factors, were defined as the sum of Log10-differences divided by the number of time points (average). Calculated hysteresis factors are shown in figs. 1C and S10.

To quantify the experienced level of hysteresis for the different antibiotic sequences in the evolution experiment, we counted the numbers of the six different drug switches (CIP->GEN, CIP->CAR, GEN->CIP, GEN->CAR, CAR->CIP and CAR->GEN) up to a particular time point, the obtained counts were multiplied with the respective hysteresis factors, and the sum of products calculated, which was normalized by dividing by the number of transfers up to that time point. The inferred levels of hysteresis per transfer are shown in figs. 2B, 3F, 5A and S14.

Dose-response curves and drug-interaction measurements

To measure dose-response curves, bacteria were grown to exponential phase ($OD_{600} = 0.08$, 8×10^7 cfu/ml) and diluted 10-fold into antibiotic containing 96-well plates. Antibiotics were dosed in eight linearly increasing concentrations that exceeded the minimal inhibitory concentration (MIC). Concentrations were spatially randomized across the plate. Plates were incubated for 12h at 37°C and 1350 rpm shaking on microplate shakers (Heidolph Instruments, Germany; Ref. Titramax 100, 1mm orbital), after which we measured growth by optical density (OD_{600}). Obtained dose response curves were analyzed to obtain standardized concentrations that achieved 75% inhibition (IC_{75}) of final yield after subtraction of background OD_{600} . Similarly we obtained information on drug interaction, by combining varying proportions of two antibiotics in a randomized checkerboard setup. Checkerboard data is shown in Fig. S2. Results based on dose-response measurements are shown in figs. 3, 4A, 5C, S7A, and S8A, S17.

Main evolution experiment

The evolution experiment was started from six different starting cultures of *P. aeruginosa* PA14 prepared from single colonies (biological replicates). These independent starting cultures were used to take account of stochastic influences, which may bias results from evolution experiments initiated with only a single clone. The starting cultures were grown to exponential phase ($OD_{600} = 0.08$) and diluted to starting cell densities of 8×10^5 cells for the first treatment step. Each biological replicate was divided into 2 technical replicates, to take account of stochastic variation during the experiment, yielding a total of 12 replicates per treatment combination. Antibiotics were dosed to

achieve 75% inhibition (IC_{75}) of final yield in this first treatment step and concentrations were constant for the whole experiment (CIP 40 ng/ml, GEN 500 ng/ml, CAR 50 μ g/ml). Every 12h we transferred 2% of the population to fresh media, containing the same or a new antibiotic. In permissive conditions, 12h are sufficient to reach carrying capacity, and populations that reach carrying capacity grow 5.6 generations between transfers. The experiment was performed in 96-well plates (Greiner Bio-One, Germany; Ref. 655161) that were incubated in plate readers (BioTek Instruments, USA; Ref. EON) with 180rpm double orbital shaking at 37°C for 96 transfers of 12h during which we measured OD_{600} every 15 min. The thus obtained time-series of growth curves is a high-resolution image of the evolutionary dynamics. We prepared a fossil record by freezing plates to -80°C every 12 transfers after adding the cryo-protectant DMSO at 10% (v/v).

In this way, we evolved 190 populations: 16 treatments x 12 replicates each, except for the no-drug reference #4, for which we had only 10 replicates in favor of empty wells in the microtiter plates for background subtraction. Treatments were systematically randomized and evenly split across two 96-well plates. Material preparation and transfers were done in a sterilized laminar flow hood. Treatment IDs were coded to exclude observer bias.

Before the experiment, we tested for the existence of spatial gradients in the plate readers. We discovered a gradient for aminoglycosides (GEN and streptomycin), but none for CIP, CAR or growth without antibiotics. The aminoglycoside-caused gradient only occurred in columns 7-12. Treatment by GEN was therefore always carried out in the unaffected half of the plate (columns 1-6).

As a measure for treatment efficacy we calculated the integral of the growth curve (area-under curve, AUC) divided by the integral for the untreated reference evolving in parallel (relative AUC; 'Relative biomass' in Fig. 2C). Low values denote sensitivity to treatment, whilst a value of 1 represents un-inhibited growth. Relative AUC is more sensitive and reproducible than endpoint population sizes or growth rates (6, 15, 26, 27) because it contains information of all growth phases. Data is shown in Fig. 2C and Fig. S5.

To evaluate the rate of the long-term evolutionary adaptive response, we calculated rates of biomass increase using a sliding window approach. Rate of biomass increase was defined as the X^{-1} , where X is the transfer at which the mean relative AUC of a sliding window of 12 transfers reaches 0.75 for the first time. This measure is related to the previously defined 'rate of adaptation' (28), which was originally defined for constant environments, yet not applicable to adaptation in fluctuating environments, in which growth often oscillates in parallel to antibiotic switching. Our measure is less biased by such oscillations and thus more broadly applicable, including the here used fluctuating environments. Calculated rates of biomass increase are shown in Fig. S6.

Frequencies of population extinction were determined after transfer 96 by counting the cases in which no growth was observed after inoculation of drug-free media and incubation for 24h. Extinction frequencies are shown in Fig. 2D.

Mathematical model

We developed a deterministic model to explore the ability of different sequences of drugs to limit increase of population size by evolution of resistant types. We made use of a modified version of a logistic growth model (competition for space) that included mutation,

$$\dot{x}_i = \left(r_i x_i + \sum_{j=1}^n (q_{ji} r_j x_j - q_{ij} r_i x_i) \right) \left[1 - \frac{1}{K} \sum_{l=1}^n x_l \right].$$

The equation refers to the typical logistic growth of different bacterial genotypes, with density x_i and growth rate r_i . Each genotype has three growth rates, for each of the possible treatments (CIP, GEN and CAR), $r_i = \{r_i^{CIP}, r_i^{GEN}, r_i^{CAR}\}$. The mutation rate denoted by q_{ji} allows the change of genotypes j to another genotype i . The carrying capacity is defined by K . To simulate serial transfers the mixture of types is diluted by a dilution factor DF at the end of each season. If the density of a genotype falls below the cutoff k during dilution, it is lost and can only reappear via mutation. Following dilution, treatments can either switch in any of the following directions (CIP->GEN, CIP->CAR, GEN->CIP, GEN->CAR, CAR->CIP and CAR->GEN) or be repeated (CIP->CIP, GEN->GEN, and CAR->CAR).

The model was parameterized to fit the conditions in the main evolution experiment. $K = 10^8$ cells, $DF = 50$ applied every 12h, $k = 10$. Population size is $K/4$ (IC_{75}) directly before the first transfer. Using this model, we generated growth dynamics for a simple system with four competing genotypes, the non-resistant *wt* and three mutants. The mutants are individually resistant to CIP, GEN, or CAR, and are parameterized according to the measurements of evolved bacterial isolates from the mono-treatments ##1-3 (isolates 12-1a-G8-3, 12-1b-B2-8 and 12-1b-E8-3 for CIP^R, GEN^R and CAR^R, respectively; see Fig. 3A for an overview of evolved resistances under monotherapy), as indicated in the following table *R*:

Growth rate table *R*

Genotype	CIP	GEN	CAR
<i>wt</i>	0.350	0.346	0.316
CIP ^R	0.504	0.072	0.145
GEN ^R	0.443	0.494	0.287
CAR ^R	0.463	0.234	0.491

Some mutant growth rates were lower than those of the *wt* on particular antibiotics, denoting collateral sensitivity, and consistent with our previous findings (26). Higher mutant growth rates denoted resistance and cross-resistance.

Switches between antibiotics allowed for hysteresis effects, which we included in the model by multiplying the respective growth rates from table *R* with the corresponding entry from the hysteresis landscape table *H*, experimentally inferred for *wt* and the individual resistant mutants (figs. S10, S11).

Hysteresis landscape table H

	Switch of treatments								
	CIP->CIP	GEN->CIP	CAR->CIP	CIP->GEN	GEN->GEN	CAR->GEN	CIP->CAR	GEN->CAR	CAR->CAR
<i>wt</i>	1	1.63	1.18	0.15	1	-0.36	1.09	1.15	1
CIP ^R	1	1.05	1.26	0.62	1	-0.21	1.47	1.37	1
GEN ^R	1	0.94	1.14	0.96	1	1.22	1.01	1.12	1
CAR ^R	1	0.85	1.01	1.05	1	1.42	0.99	0.99	1

Using this model, we generated growth dynamics for mixed populations for the different treatment sequences of the evolution experiment (Fig. 2A). We re-modeled the growth dynamics with a derivate model, in which matrix H was replaced by multiplication with 1, thereby excluding hysteresis effects. From the modeled dynamics we inferred i) the strength of selection, using the cell density of the sensitive *wt*, and ii) the within-population diversity, as calculated from Shannon entropy.

The modeled growth dynamics are shown in Fig. S12 and Fig. S13, inferred strength of selection in Fig. S12, and within-population diversity in Fig. 3G.

Resistance measurements for evolved populations.

Populations were characterized after transfers 12 and 48, because the variation in evolutionary dynamics among treatments was most pronounced just before transfer 12 and then gradually decreased until they became insignificant after approximately transfer 48 (Fig. 2C). Evolved populations were thawed, mixed and plated on LB plates after serial dilution in PBS. Plates were incubated for 30h at 37°C to enable appearance of slow-growing colonies. Isolates were picked in an unbiased way: before picking, the plate was sectored, and 20 (for the initial screen) or five (for the extension of the analysis) colonies were labeled for picking using a randomized scheme. Due to the high number of colonies, we restricted resistance measurements to a subset of populations, as described below.

Initial screen: For feasibility, we focused on one population descended from a specific starting culture (culture 'b'), which was selected because it had surviving descendent populations for all sequences. We randomly chose one of the two technical replicates per biological replicates for strain isolation. The high number of isolates for each population allowed us to assess the phenotypic within-population diversity. In brief, we used hierarchical clustering to identify phenotypic sub-populations that differed in resistance profiles (see Statistics for details). The clustering yielded 1-4 clusters per population, with different frequencies (Fig. 4C). The isolate counts for the clusters were used to calculate the total within-population diversity using Shannon entropy ('Shannon diversity').

Extension of the analysis: We randomly chose 48 additional populations from the other starting cultures (3 per sequence). We avoided to sample descendants from starting culture 'f' (extinction of all populations from sequence #4) and 'a' (generally very high extinction frequencies across sequences). Due to extinction and the restriction by starting cultures, we only tested two additional populations for sequences #11 and #16, and one additional lineage for #7.

We measured antibiotic dose-response curves for a total of 880 evolved isolates on CIP, GEN and CAR. Isolates were grown in 2ml M9, diluted to standard cell density ($OD_{600} = 0.08$) and added to 96-well plates containing eight concentrations of antibiotic from 1/8 MIC to 16x MIC. Treatment combinations were all randomized systematically. On each plate, we included two controls of the ancestral strain *P. aeruginosa* PA14. Plates were incubated for 12h at 37°C and 1350 rpm shaking on microplate shakers (Heidolph Instruments, Germany; Ref. Titramax 100, 1mm orbital), after which we measured growth by optical density (OD_{600}). Resistance values were calculated as the area between dose-response curves of the isolates and those of the PA14 controls measured on the same plate. A resistance value of zero denotes no resistance, negative values denote hypersensitivity and positive values denote resistance. The advantage of this value as compared to MIC is that it is a continuous variable rather than an ordinal variable.

To compare the efficacy of different treatments using a single value we defined multidrug resistance scores (MDR) as the sum of resistance values on the three antibiotics. MDR scores represent the distance to the sensitive strain in a three-dimensional resistance space with axes for CIP^R, GEN^R and CAR^R.

Before the assay, we checked for the existence of spatial gradients in the incubators. We found no spatial gradients for fluoroquinolones, beta-lactams or growth without antibiotics. However, we found a spatial gradient for aminoglycosides. The gradient occurred between different shelves in the incubator, but not within one shelf. We controlled for this gradient, by always incubating plates of a given antibiotic on the same shelf.

Resistance data of the initial screen is shown in figs. 3A, 3B, and 3E. Calculated MDR including the additional samples from the extension of the analysis is presented in figs. 3E, 3F, and 4A. Diversity data is shown in Fig. 3C.

Measurements of growth rates under drug-free conditions

We measured maximum exponential growth rates of the sub-populations from the initial resistance screen under drug-free conditions. For feasibility, growth rates of sub-populations were determined with representative isolates (1-4 isolates per population and time point). The values reported in Fig. 3D represent the mean of the measured isolates, weighted by the relative proportions of the respective sub-populations. Growth rates were calculated from growth curves obtained in 96-well plates, using BioTek plate readers. Prior to measurements, we sub-cultured the samples in the plate readers for 16h to allow them to adjust to assay conditions. Immediately after end of incubation, cultures were diluted 1000x and re-incubated for 24h during which we measured OD_{600} every 15 min (measurements in triplicate). Sample positions were randomized. Reference cultures of *P. aeruginosa* PA14 were included in every run. Maximum exponential growth rates were determined using a sliding window approach. We calculated specific growth rate k in sliding windows of size 0.5h, yielding hill-shaped curves with two peaks, a first peak for growth on glucose and a second peak for growth on citrate (figs. S7 and S8). The growth rates reported in Fig. 3D are the maximum values of the first, larger peak. This procedure was found to yield more reproducible results than measurements in defined OD_{600} -windows. Prior to the experiment, we checked for the existence of spatial gradients, by measuring exponential growth rate for PA14. Spatial gradients were small, but we nevertheless created a function to correct systematic error. The inferred growth rates are shown in figs. 3D, S7, and S8.

Genomics

We whole-genome sequenced 30 evolved isolates from different sub-populations as identified by the resistance measurements of the initial screen. We included all sub-populations for the early time point, and the 3 sub-populations of sequence #8 at the late time point. DNA was isolated from 5ml cultures that were prepared from single colonies with a cetyltrimethylammonium bromide-based protocol (29). To verify that the colonies had the previously measured resistance profile, we repeated resistance measurements on the cultures from which DNA was extracted (Fig. S9). Sequencing was performed by the Institute for Clinical Molecular Biology (ICMB) Kiel, using the Illumina MiSeq 2x150bp paired-end technology and NexteraXT library preparation.

Paired reads were filtered with *Trimmomatic* (30) and mapped against the UCBBP_PA14 reference genome (31) with *Bowtie2* (32). Variants were called using the function *mpileup* from *SAMtools* (33) and filtered by *Vcftools* (MinQ = 40) (34). We subtracted all mutations already present in our previously sequenced lab strain (26). Gene annotations were improved by BLASTing genes against the better annotated reference genome of *P. aeruginosa* PAO1. Additional annotation of variants was done with customized python scripts. Intergenic regions containing mutations were analyzed for the presence of promoter motifs using PePPER (35).

All intergenic mutations outside of promoter regions were excluded from analyses. Samples shared mutations in the genes PA14_61200 and PA14_38000, both annotated 'hypothetical protein'. These mutations were excluded from analyses. The resulting list of variable sites is provided in Table S1 and illustrated in Fig. S15 and Fig. S16.

Annotation of gene PA3206 (PA14_22730) as a new two-component sensor “cpxS”

Mutations in the previously uncharacterized gene PA3206 (PA14_22730) were indicated to contribute centrally to the evolutionary response to negative hysteresis (Fig. 4). Therefore, we sought to obtain a more detailed understanding of the function of this gene. We started by analyzing its genomic context in *Pseudomonas aeruginosa*. PA3206 is in close proximity to PA3204 and PA3205 (Fig. S18A), which have recently been annotated “cpxR” and “cpxP”, respectively (36). CpxR forms a two-component regulatory system with the sensor CpxA that is negatively regulated by CpxP in *Escherichia coli* (20) (Fig. S18C). In *E. coli* it is activated by misfolded proteins, as generated by aminoglycosides (19), and CpxA-CpxR is involved in intrinsic resistance to these drugs (20). The genomic location suggested that PA3206 may be a homolog of cpxA. However, sequence identity between CpxA from *E. coli* MG1655 (NCBI Gene [uid] 948405) and PA3206 from PA14 was only 30%. We BLASTED the protein sequence of CpxA from MG1655 against all proteins in the NCBI database from *P. aeruginosa*. Alignments only started at residue 160, indicating differences in the N-terminal sensor domain. Indeed, the best hits were ParS, a different two-component sensor, suggesting that PA3206 is distinct from CpxA. This was confirmed by *phmmmer* (37) analysis (aligning CpxA from MG1655 against *P. aeruginosa* proteins): the N-terminal periplasmic sensor domain has different structure, and also differs with respect to length, the number of transmembrane domains and the presence of a signal peptide (Fig. S18B and S18E). This line of evidence was further confirmed by a phylogenetic sequence comparison of PA3206 with all genes that were annotated “cpxA” in the NCBI Gene database (search “cpxA[sym]”) using *clustal-omega* (38). We used protein sequences of the closely related two-component sensor “EnvZ” as outgroup (Fig. S18D). We conclude that PA3206 is a new gene, which we here name “cpxS”. We decided to keep the base-name due to its genomic context. The “S”

denotes that it is the sensor-component of a putative envelope stress-system CpxS-CpxR in *P. aeruginosa*. The *de-novo* mutations in this study targeted the putative periplasmic sensor domain (Fig. S14F), which showed very little resemblance to the canonical sensor *cpxA* in *E. coli*, *Salmonella enterica*, *Yersinia pestis* and *P. fluorescens* (Fig. S18B and S18E), possibly indicating a currently unknown sensor function.

Re-construction of mutations

Slow-regular cycling was associated with mutations in *mexR*, and fast-regular cycling was associated with mutations in *cpxS* (homolog of PA3206 in reference strain PAO1). For functional characterization, we chose one variant of each gene: mutation 486113T>G = *mexR* c.388T>G p.T130P (sequence #10 isolates 12-1b-A2-4; #10-1 in Fig. 5B; and 12-1b-A2-8 ; #10-2 in Fig. 4B) and mutation 1977519T>G = *cpxS* c.487T>G p.T163P (sequence #7 isolates 12-1b-F2-4; #7-1 in Fig. 4B; and 12-1b-F2-3; #7-2 in Fig. 4B). The two mutations were individually introduced into the genetic background of the ancestral *P. aeruginosa* PA14 strain using a scar-free recombination method (39). The work was performed by V. Trebosc and C. Kemmer from the company BioVersys AG (Hochbergerstrasse 60c, CH-4057 Basel, Switzerland). Mutations were confirmed by Sanger sequencing.

Measurement of hysteresis in mutants

Exponential phase cultures were prepared and pre-treated as described in section “Time-kill experiments and hysteresis measurements”.

Growth rate: Cultures were diluted 1:10 into 96-well plates and growth curves in the presence of GEN 420 ng/ml (sub-lethal) were acquired with BioTek plate-readers (37°C, 180rpm orbital shaking, OD₆₀₀ every 15 min for 20h). Growth dynamics are shown in Fig. 4C.

Flow cytometry: Cells were re-suspended in GEN 420 ng/ml (sub-lethal) and incubated at 37°C. Samples were taken in hourly intervals and population survival was assessed using Live/Dead staining with the fluorescent dyes Propidium iodide 12.5 µg/ml (Sigma-Aldrich, USA; Ref. P4170-25MG) and Thiazole Orange 0.4 µg/ml (Sigma-Aldrich, USA; Ref. 390062-250MG). For staining, cells were diluted 25x and incubated in PBS containing the dyes for 10 minutes. Samples were then diluted 40x and the proportion of dead cells was scored by flow cytometry (Guava EasyCyte HT Blue-Green, Merck KGaA, Darmstadt, Germany). Thresholds were set using side scatter (SSC = 4) and 5000 events were acquired for each sample using low flow rate 0.24µl/s. Counting gates were set in green and red fluorescence channels. Samples were measured with three technical replicates. Flow cytometry data is shown in Fig. 4D.

Measurement of tolerance

Antibiotic tolerance was assessed for isolate 12-1a-E2-4 (isolated after transfer 12 from sequence #12) via minimal duration of killing (MDK), as previously described (17). MDK90 and MDK99 values were obtained from time-kill experiments with CIP 50 ng/ml, GEN 500 ng/ml and CAR 100 µg/ml. Time-kill data und calculated MDK-values are presented in Fig. 4B and Table S3, respectively.

Replay evolution experiment and resistance measurements of evolved populations

The replay evolution experiment was conducted as described for the main evolution experiment, except that it was started from single starting culture of *P. aeruginosa* PA14 and lasted 12 transfers only, as we were interested in the clinically relevant phase of the adaptive dynamics. We included 12 replicates for each of five treatment groups: untreated references, sequences #12, #12rev, #13 and #13rev. Sequences #12 and #13 were the same as the first 12 transfers in the main experiment, and sequences #12rev and #13rev were their respective reverse sequences. Sequences #12 and #13 were selected for this experiment because they differed in their level of hysteresis and produced different levels of MDR in the main evolution experiment. Importantly, the sequences individually started and ended with the same antibiotic.

Frequencies of population extinction were determined after transfer 12 by counting the cases in which no growth was observed after inoculation of drug-free media and incubation for 24h. Resistance of all surviving evolved populations was measured as described above after sub-culturing in 5ml M9 (inoculated with 20 μ l of the frozen populations) and with two technical replicates. Resistance was quantified by the fold-changes in IC₇₅. To assess whether reversing drug order altered resistance levels, we calculated the relative change in mean resistance for the two sequence pairs (fold-IC₇₅FORWARD/fold-IC₇₅REVERSE \times 100%). Calculated resistance changes and extinction frequencies reported in Fig. 5B and Fig. 5C.

Statistical Analysis

Data analysis and statistics were performed with the statistics software R (40). Additional information of the statistics is provided in the accompanying Excel file, which contains a separate sheet for each of the performed statistical analyses.

Evolutionary dynamics: The efficacy of cycling strategies to constrain adaptation was statistically assessed using mixed linear models (GLMM, R package 'nlme' (41)) with relative AUC (see methods; 'Relative biomass' in Fig. 2C) as response variable, sequence and transfer as fixed factors, and starting culture and population as random factors nested in treatment. Due to extinction and populations that started growing late (putative persisters) relative AUC had a bimodal distribution (Fig. S3). We removed bimodality by excluding extinct and persister populations from the analysis. Thereby, our analysis is statistically conservative, because extinction and persister frequencies were higher in fast-regular and random treatments. We divided analysis for three phases of the evolutionary dynamics: 'early' = transfers 1-12, 'mid' = transfers 13-48 and 'late' = transfers 49-96. This splitting was necessary because the evolutionary dynamics were non-linear. It removed structure from model residuals, which were normally distributed (Fig. S4). *P-values* were obtained from *post hoc* tests (function 'glht' from R package *multcomp* (42)) and corrected for multiple testing using false-discovery rate (FDR).

Extinction: We tested for significant differences in extinction frequencies between random, fast-regular and slow-regular cycling using Fisher's exact test and total counts of extinct and surviving populations for the cycling strategies.

Identification of sub-populations: Sub-populations were identified by hierarchical clustering of the resistance profiles of isolates from the same population and time point with the R package *pvclust* (43): method = 'average', 'euclidean' distances. Different clustering algorithms ('median', 'ward.D2') yielded the same clusters. This analysis identified groups of isolates that share dose-

responses that are correlated between antibiotics. Isolates were added to a new cluster if they had a height larger than the threshold of $h = 0.2$. After applying clustering with this threshold, the residual variation in dose-response curves was lower than that of the isogenic strain PA14.

Population diversity: We tested whether populations from monotherapies, the no-drug reference, random, fast-regular, and slow-regular cycling (all pair-wise comparisons) had significantly different within-population diversities (Shannon entropy) using GLMM and *post-hoc* tests. The model had Shannon entropy as response variable, sequence as fixed factor and time point as nested random factor. We tested whether there were differences in diversity between random and fast-regular cycling and between random and slow-regular cycling, using the same model. *P*-values were adjusted for multiple testing by FDR.

Growth rate under drug-free conditions: We tested whether there were significant differences in relative exponential growth rates between populations from random and fast-regular cycling, slow-regular cycling (all pair-wise comparisons) after transfers 12 and 48 using GLMM and *post-hoc* tests. The model had relative exponential growth rate as response variable, sequence as fixed factor and population as nested random factor. *P*-values were adjusted for multiple testing by FDR.

Multidrug resistance: We tested for significant differences in MDR after transfer 12 between random, fast-regular and slow-regular sequences using GLMM and *post-hoc* tests. The model had MDR as response variable, sequence as fixed factor and population as nested random factor. *P*-values were adjusted for multiple testing by FDR. We also tested whether MDR in sequence #12 was significantly different from that of the ancestor using GLMM and *post-hoc* tests. The model had resistance as response variable, treatment group (ancestor or sequence #12) and antibiotic as fixed factors and population as nested random factor.

Overrepresentation of mutational targets: We statistically identified genes that were over-represented among sequenced isolates from slow-regular or fast-regular cycling using Fisher's exact test and counts for presence and absence of mutations in a focal gene from isolates.

Resistance in re-play evolution: Fold-changes in IC_{75} were calculated from dose-response curves. We tested for significant differences in evolved resistance between sequences 12-12rev and sequences 13-13rev using GLMM and *post-hoc* tests. The model had fold-change IC_{75} as response variable, sequence as fixed factor and antibiotic as random factor.

Supplementary Figures

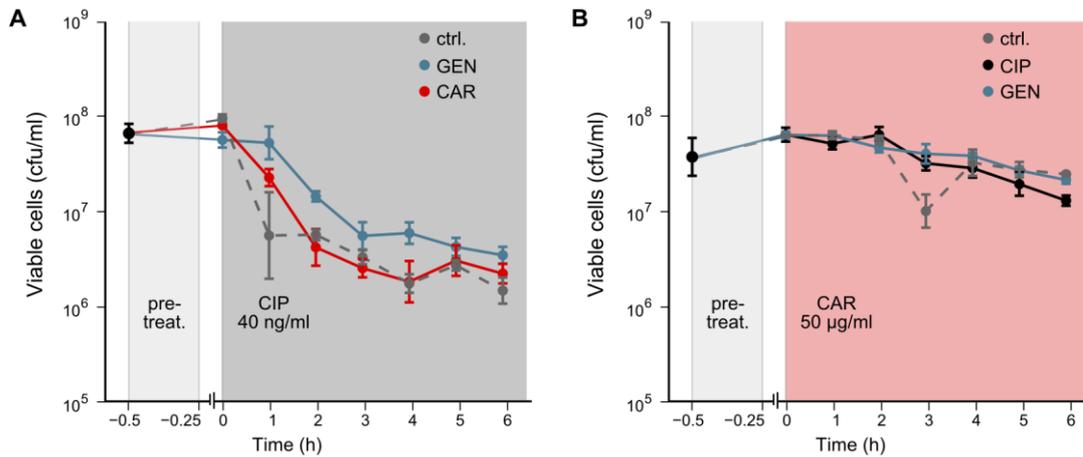


Fig. S1. Short antibiotic exposures can inhibit killing by antibiotics in *Pseudomonas aeruginosa*. (A) Time-kill experiment with ciprofloxacin after 15min pre-treatments with non-lethal concentrations of specified antibiotics. (B) Time-kill experiment with carbenicillin after 15min pre-treatments with non-lethal concentrations of specified antibiotics. CIP, ciprofloxacin; GEN, gentamicin; CAR, carbenicillin; ctrl, control without pre-treatment; n = 6 technical replicates; bars represent mean ± SEM.

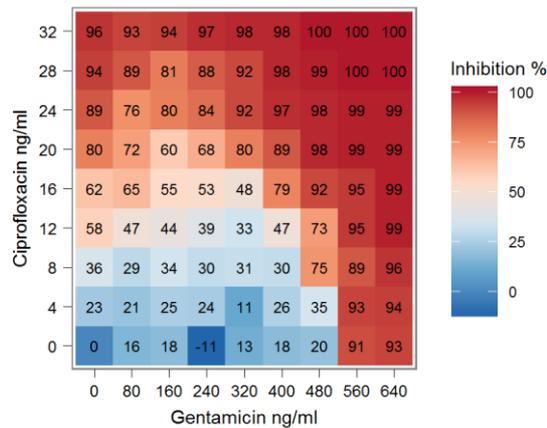


Fig. S2. Antagonistic drug-interaction (directional suppression) between ciprofloxacin and gentamicin in *Pseudomonas aeruginosa*. Values denote mean inhibition of growth, as determined by measurements of optical density (OD₆₀₀) after 12h. Concentrations were spatially randomized in the checkerboard experiment. n = 3 technical replicates.

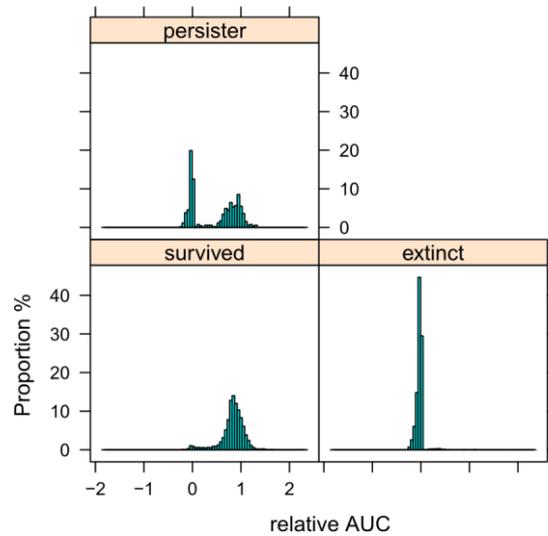


Fig. S3. Distribution of response variable for GLMM of evolutionary dynamics. Response variable relative biomass (relative AUC) showed a bimodal distribution. The bimodality was removed by only including survived populations in the statistical analyses.

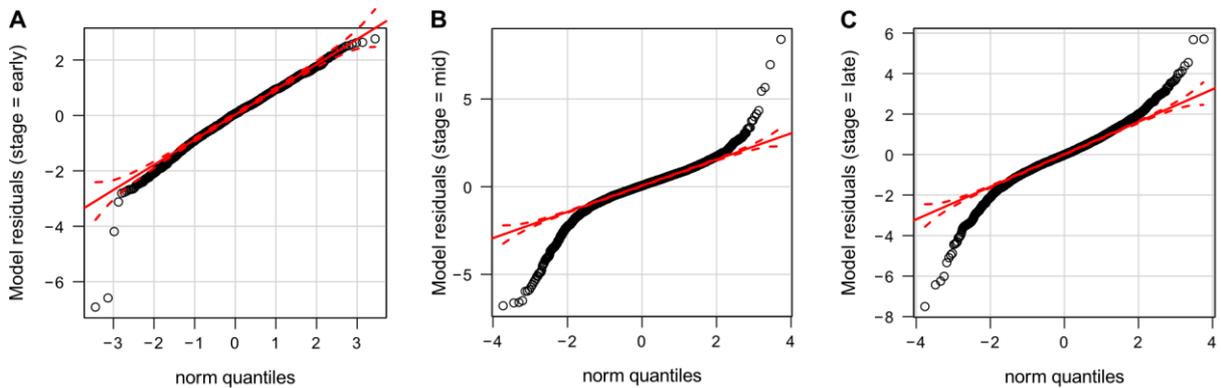


Fig. S4. QQ-plots of model-residuals from GLMM of evolutionary growth dynamics as reported in Fig. 2C and statistics file sheet 1. (A) Pearson model residuals of all treatments for early stage (transfers 1-12). (B) Pearson model residuals of all treatments for middle stage (transfers 13-48). (C) Pearson model residuals of all treatments for late stage (transfers 49-96).

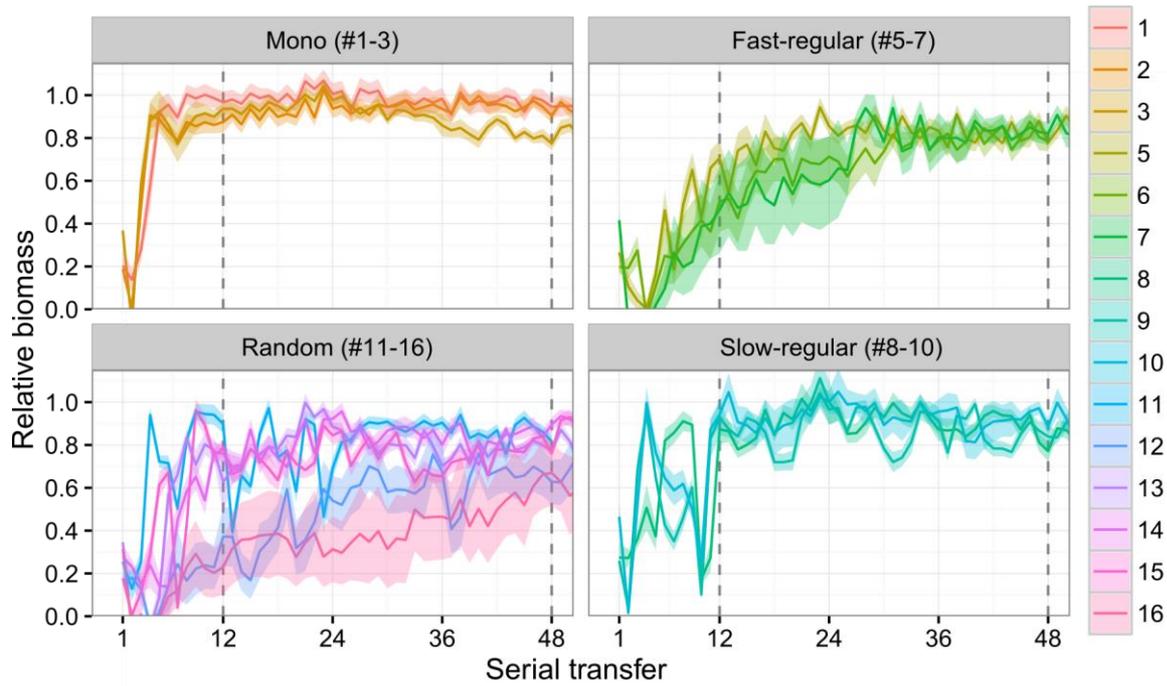


Fig. S5. Variation in adaptive dynamics between sequences. In general, relative biomass increased the fastest in monotherapies and slow-regular sequences and less quickly in fast-regular sequences. There is pronounced variation between random sequences, which includes the slowest adaptation. Extinct populations were excluded. Lines show means of surviving populations ($n = 4-12$ biological replicates) and shading denotes \pm SEM.

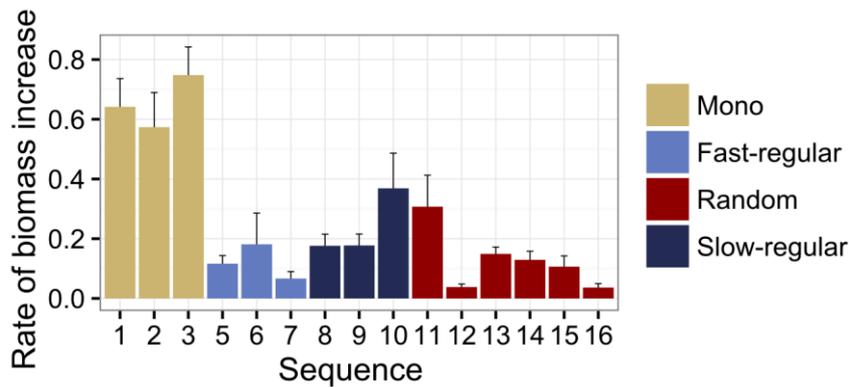


Fig. S6. Variation in the rates of biomass increase among sequences. The rate of biomass increase is defined as X^{-1} , where X is the first transfer at which the mean relative biomass in a sliding window of 12 transfers increases to 0.75. See methods for details. Extinct populations excluded.

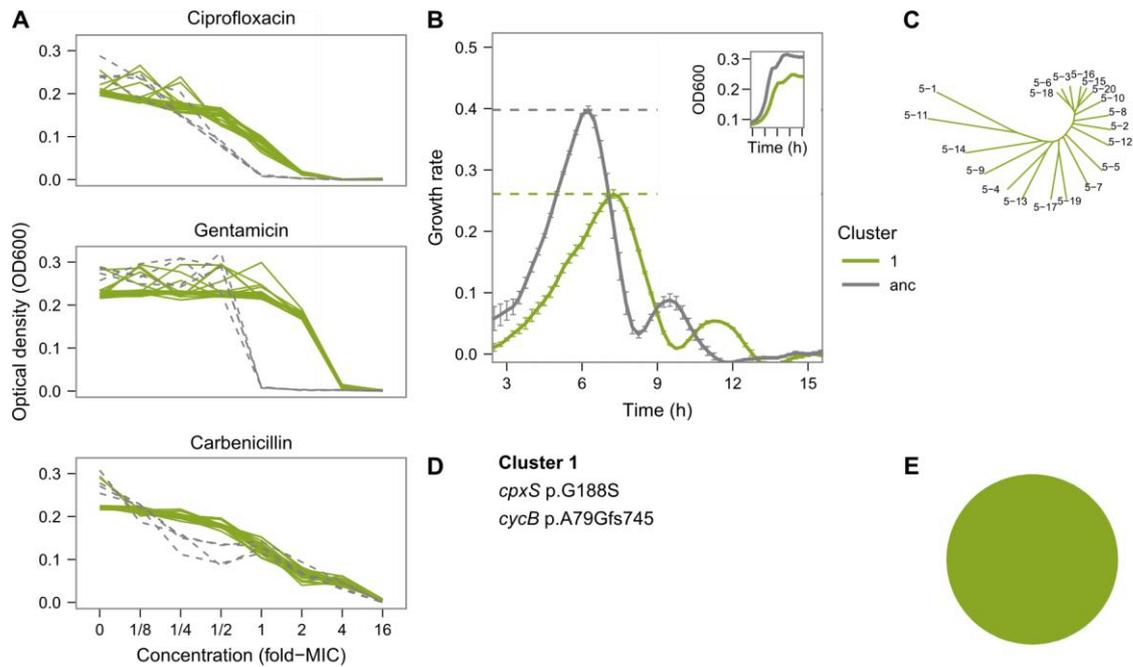


Fig. S7 Raw data from characterization of population 12-1a-D8 from fast-regular sequence #5. (A) Dose-response curves of 20 isolates and 4 ancestral controls. Isolates showed homogenous resistance profiles with low-level resistance. (B) Maximum exponential growth rate in media without antibiotics is decreased substantially, as determined using a sliding window approach. Inlay shows growth curve. Bars represent mean \pm SEM, $n = 3$ technical replicates. (C) Dendrogram with results of hierarchical clustering. (D) Evolved genetic changes, as determined from whole-genome sequencing. (E) Plot indicating the relative frequency of different sub-populations. In this case, the clustering analysis identified only a single sub-population.

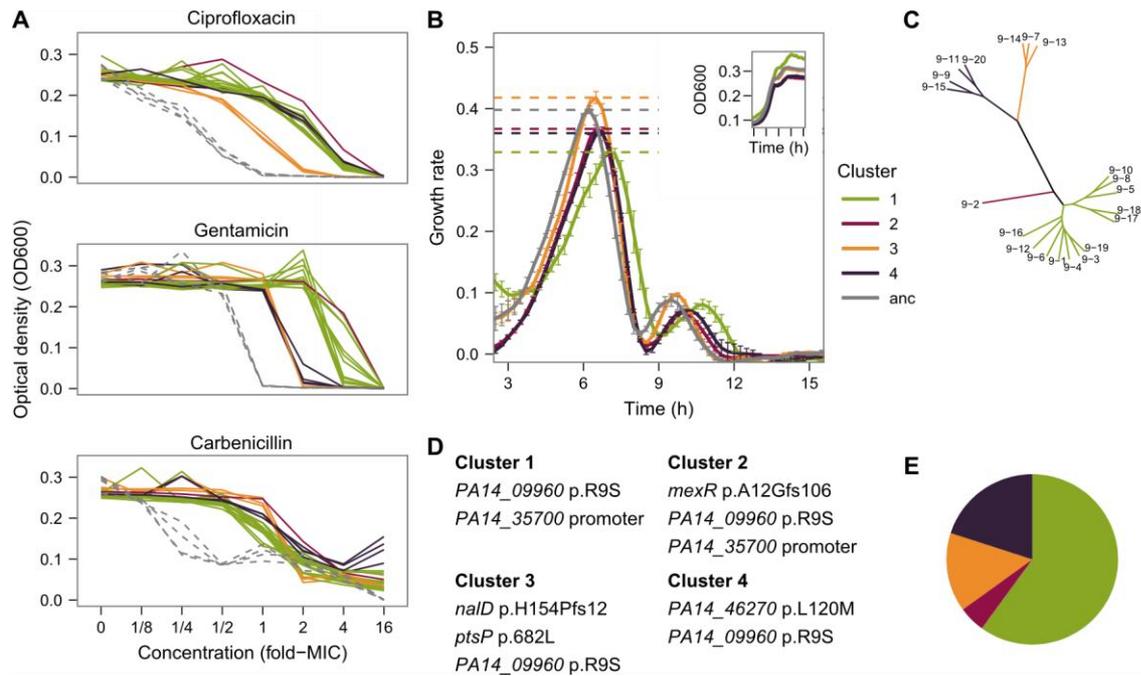


Fig. S8. Raw data from characterization of population 12-1b-A8 from slow-regular sequence #9. (A) Dose-response curves of 20 isolates and 4 ancestral controls. Substantial between-isolate variation is found for resistance profiles. (B) Maximum exponential growth rate in media without antibiotics indicates larger fitness costs in the green sub-population, as determined using a sliding window approach. Inlay shows growth curves. Bars represent mean \pm SEM, $n = 3$ technical replicates. (C) Dendrogram with results of hierarchical clustering, indicating presence of four clusters. (D) Genetic differences of clusters, as determined from whole-genome sequencing. (E) Relative frequency of the four sub-populations.

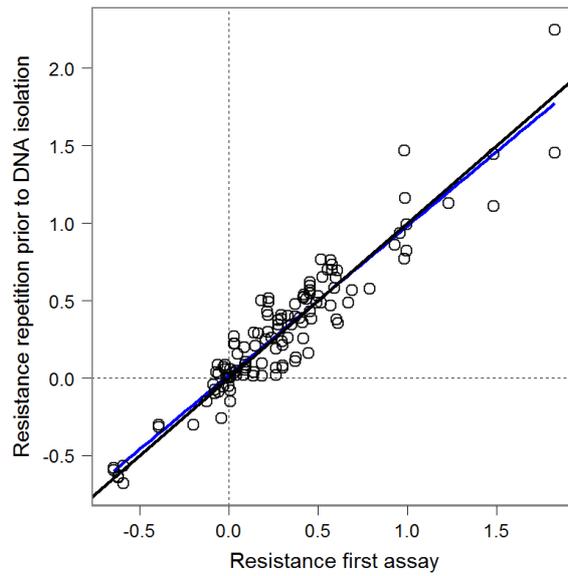


Fig. S9. Repeatability of resistance measurements. The measurements of resistance are highly reproducible: Linear model: $y = f(x) = 0.96063x + 0.01916$. $R^2 = 0.89$. $P < 2.2E-16$ (Pearson product-moment correlation, $t = 30.818$, $n = 120$ biological replicates). Blue, linear model; black, diagonal with slope 1.

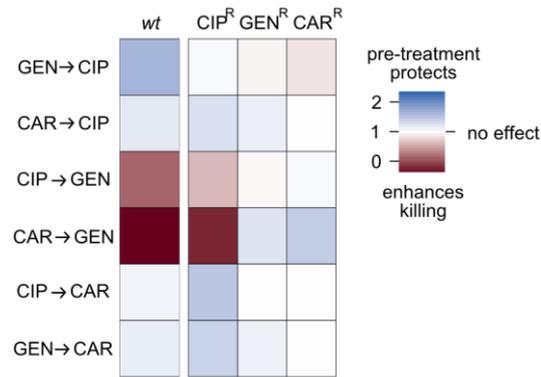


Fig. S10. Extension of hysteresis matrix for resistant genotypes from monotherapies. Hysteresis factors determined from time-kill experiments for evolved isolates resistant against ciprofloxacin, (CIP^R), gentamicin (GEN^R) or carbenicillin (CAR^R). A superscript ^R denotes resistance against the respective antibiotic. CIP, ciprofloxacin; GEN, gentamicin; CAR, carbenicillin. The values are included in the mathematical model as shown in Fig. 3, figs. S12, S13.

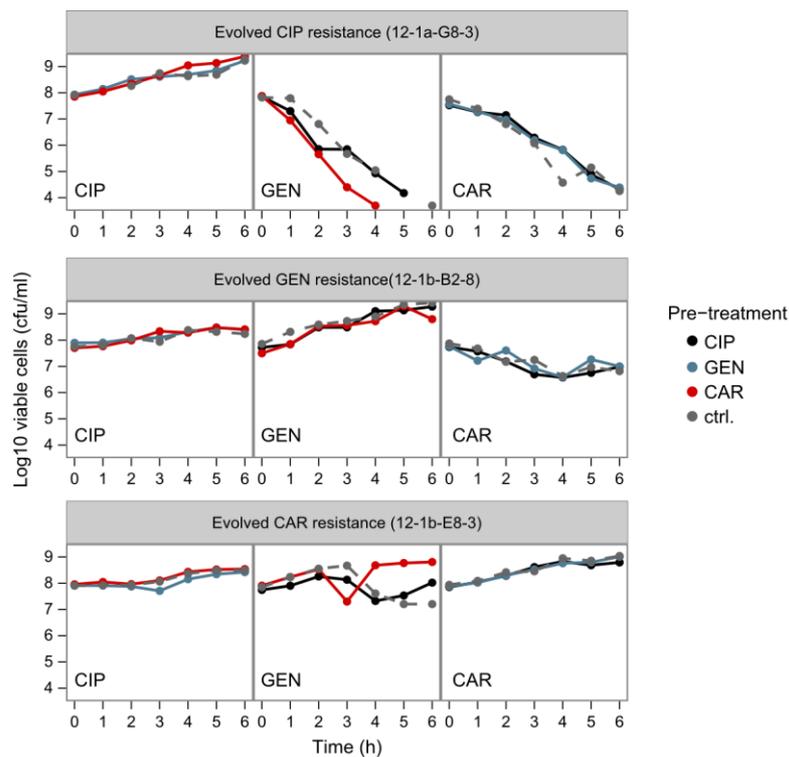


Fig. S11. Time-kill data of resistant types used for the calculation of the hysteresis landscape of evolved isolates from monotherapies. CIP, ciprofloxacin 40ng/ml; GEN, gentamicin 480 ng/ml; CAR, carbenicillin 50 µg/ml; ctrl, control without pre-treatment.

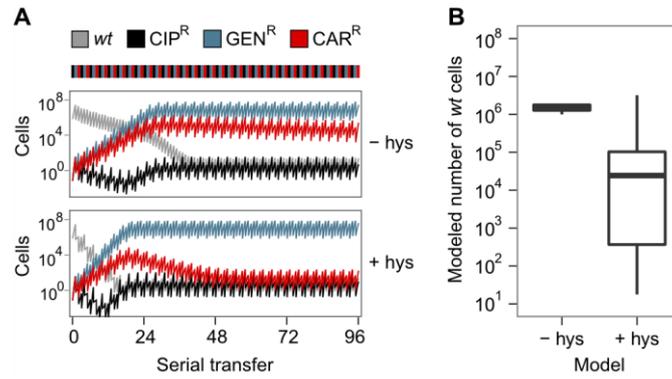


Fig. S12. Mathematical model predicts strong selection by hysteresis and reduced clonal interference in fast sequential treatments. (A) Evolutionary dynamics for fast-regular sequence #5 as produced by a deterministic model without hysteresis (- hys) or with hysteresis (+ hys). Superscript ^R, resistant type; wt, wildtype; CIP, ciprofloxacin; CAR, carbenicillin; GEN, gentamicin. (B) The strength of selection as inferred from the reduction in wt-cells is strongly increased by hysteresis.

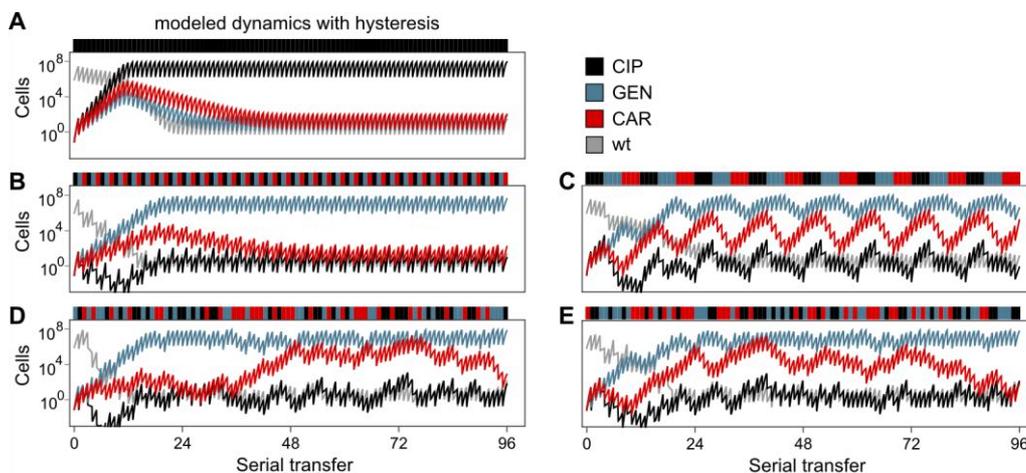


Fig. S13. Examples of modeled evolutionary dynamics for different cycling strategies. Model includes hysteresis. (A) Monotherapy sequence #1. (B) Fast-regular sequence #5. (C) Slow-regular sequence #8. (D) Best random sequence #12. (E) Worst random sequence #13. Colors denote antibiotics for the schematics and also the respective resistant types. CIP, ciprofloxacin; GEN, gentamicin; CAR, carbenicillin.

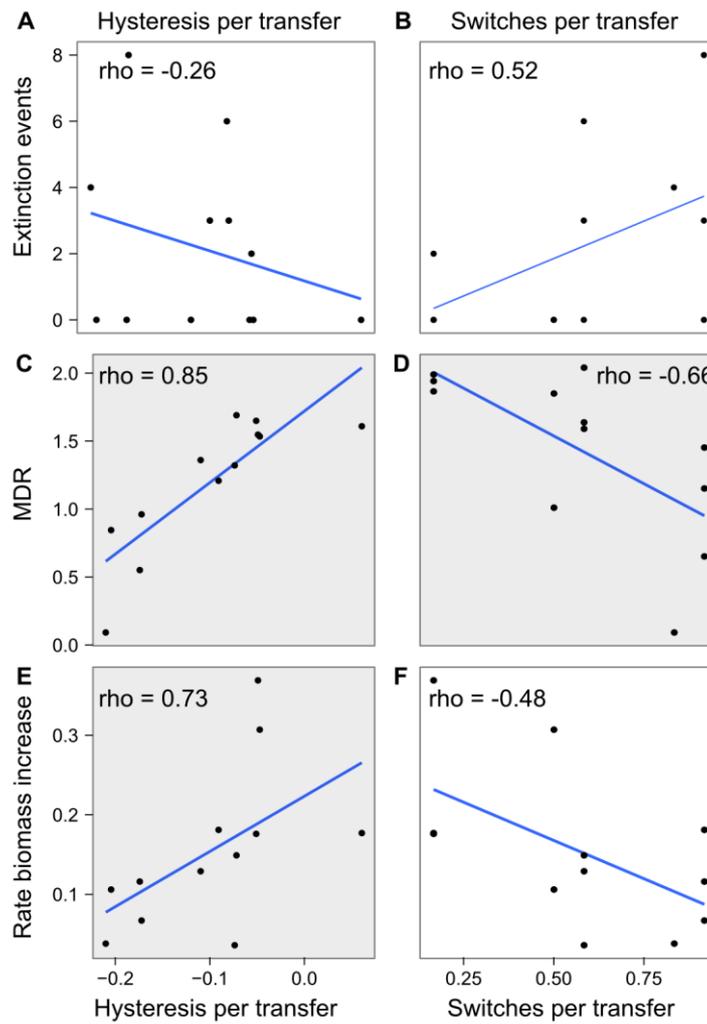


Fig. S14. Correlation between switching rate or hysteresis level with three measures of of treatment efficacy. For comparability, the correlations were restricted to all of the cycling sequences #5-16, but excluding monotherapies. The major determinants of treatment efficacy are indicated across the three rows. Shaded plots show significant Spearman rank sum correlations.

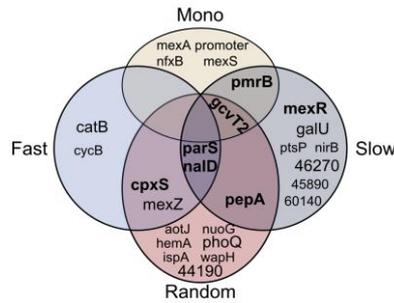


Fig. S15. Genetic basis of adaptation. Overlap of mutational targets among treatment types (as in Fig. 2A). Typeface and boldness indicate number of mutations in the gene. Genes with annotation “hypothetical protein” excluded. See Table S1 for raw data.

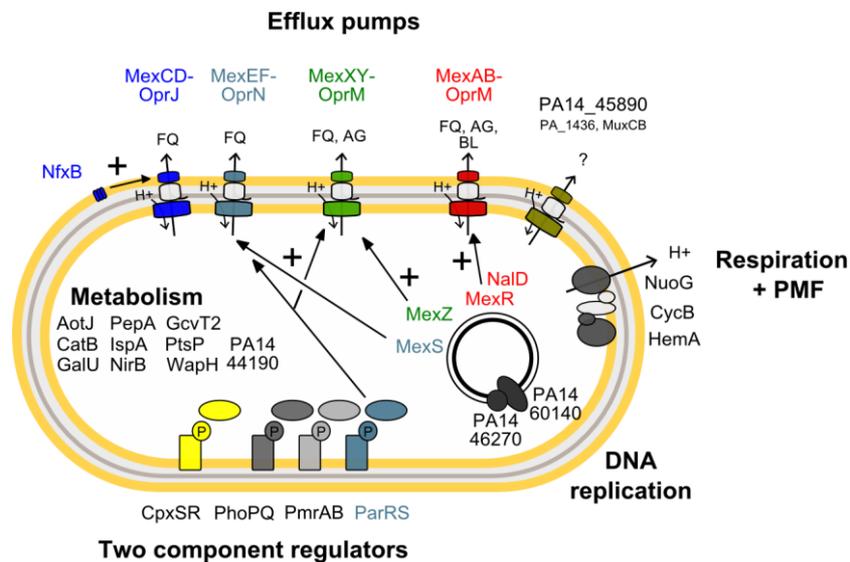


Fig. S16. Schematic of cellular functions targeted by adaptive evolution. Resistance is mostly achieved by mutations in two-component regulators or through mutations in transcriptional regulators that control efflux pumps of different substrate specificities. FQ, fluoroquinolones; AG, aminoglycosides; BL, beta-lactams; PMF, proton motive force. Further details in Table S1 and Fig. S15.

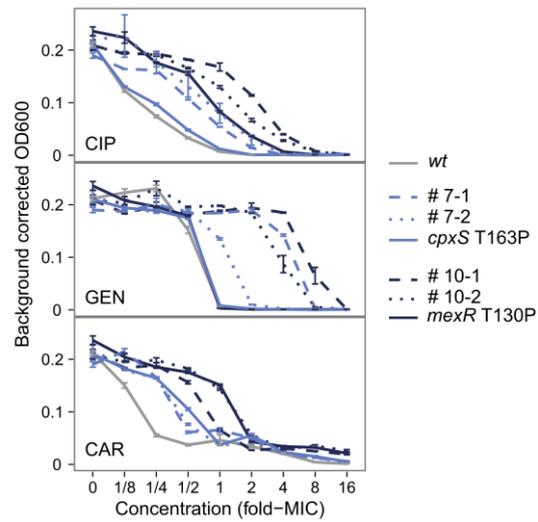


Fig. S17. Dose response curves of evolved lineages #7-1, #7-2, #10-1, #10-2 and mutants *cpxS* T163P, *mexR* T130P as shown in Fig. 4B. CIP, ciprofloxacin; CAR, carbenicillin; GEN, gentamicin; *wt*, wildtype; mean \pm SEM, n = 6-20 replicates.

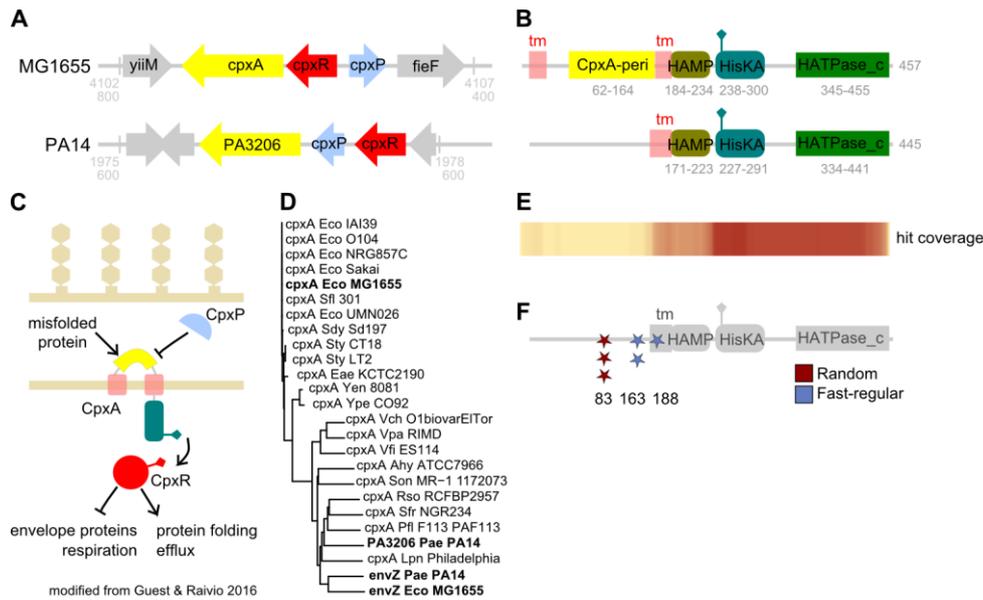


Fig. S18. Annotation of PA3206 (PA14_22730) as a new two-component sensor called cpxS. (A) Genomic context of *cpxA* in *Escherichia coli* K12 MG1655 and *PA3206* (*PA14_22730*) in *Pseudomonas aeruginosa* PA14, suggesting that *PA14_22730* may be a homolog of *cpxA*, because of proximity to *cpxR* and a small periplasmic repressor protein resembling *cpxP* (36). (B) Protein domain structure of *E. coli* CpxA and *P. aeruginosa* PA3206, as predicted by *phmmer*. The proteins differ in length, their periplasmic sensor domain, and the number of transmembrane domains. (C) Schematic of the function and regulation of the two-component regulatory system CpxAR in *E. coli* (20). In *E. coli*, the envelope stress response system CpxA-CpxR is activated by misfolded proteins, as generated by aminoglycosides (19), and CpxA-CpxR is involved in intrinsic resistance to these drugs (20). (D) Phylogenetic tree of CpxA inferred from protein sequences using *clustal-omega*. Protein sequences of the two-component sensor EnvZ serves as outgroup. (E) Alignment of *E. coli* CpxA against all proteins from *P. aeruginosa* using *phmmer* shows very low coverage for the periplasmic sensor domain. In consideration of these differences, the structural variation, and the similarity to other two-component sensors, *PA14_22730* is unlikely a true homolog of *cpxA*. (F) Mutational targets found in the evolved, genome-sequenced isolates are located in the putative sensor domain of *PA3206* (indicated by stars, while colors denote the treatment types). Because similar levels of sequence identity exist to other two-component systems, but the genomic context indicates interaction with *cpxR*, we here name this *P. aeruginosa* gene “*cpxS*”, the “S” denoting that it is the sensor component of a putative envelope stress-system CpxS-CpxR in *P. aeruginosa*.

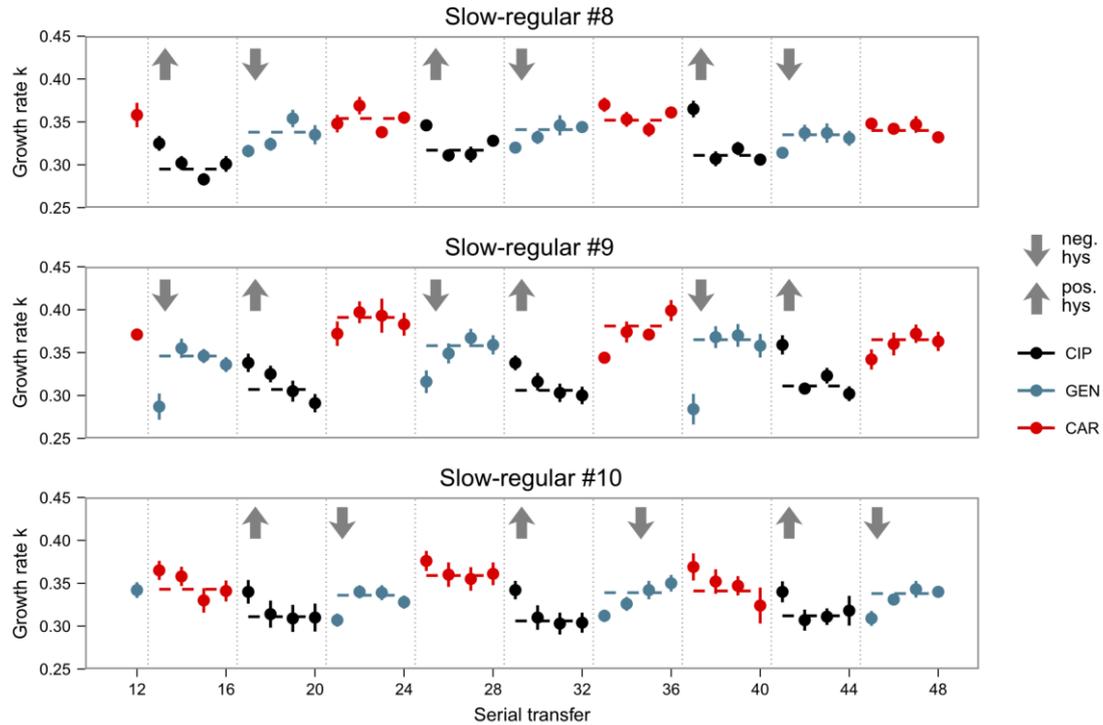


Fig. S19. Verification that hysteresis was triggered in the evolution experiment. Exponential growth rate of populations in slow-regular cycling between transfers 12 and 48. CIP, ciprofloxacin; GEN, gentamicin; CAR, carbenicillin. As predicted by the hysteresis landscape in Fig. 1C, previous exposure to either CAR or CIP transiently inhibited growth rates on GEN (i.e., negative hysteresis ‘neg. hys.’, indicated by downwards arrows). The growth rates returned to baseline levels, after one transfer. Conversely, previous exposure to CAR or GEN caused transient spikes in growth rates on CIP before returning to baseline levels, which was also predicted from the hysteresis landscape, because previous exposures to CAR or GEN protected cells from CIP (i.e., positive hysteresis ‘pos. hys.’, indicated by upwards arrows). Previous exposures did not consistently affect growth rate on CAR, which agreed with the hysteresis factors that were close to 1 for switches to CAR. $n = 10-12$ biological replicates; bars represent mean \pm SEM. Extinct and putative persister population excluded.

Table S1. Genetic changes compared to ancestral PA14 *wt* strain from whole-genome resequencing*.

Treatment	SEQ	Isolate	POS	REF	ALT	QUAL	Depth	Feature	ST	Gene ID	Product	Type	Mutation nucleotide	Mutation amino acid
Mono	1	12-1a-G8-3	165	G	A	222	29	CDS	-	ywjB	hypothetical protein	sub_syn	c.33G>A	p.A11A
			916	CT	C	214	57	CDS	+	nfxB	transcriptional regulator	indel	c.161delT	p.L54Pfs9
			818	C	CA	44	16	CDS	+	gcvT2	glycine cleavage system protein	indel	c.589insA	p.Y197Ifs232
		249	12-1a-G8-5	282	TGCTCGGCGATGT CTCCGCCACCCGC	TGC	214	18	CDS	+	mexS	oxidoreductase	indel	c.689_711del
	2	12-1b-B2-8	563	G	A	222	10	CDS	+	pmrB	two-component sensor	sub_non_syn	c.983G>A	p.R328H
			766	G	A	193	9	CDS	+	PA14_60000	hypothetical protein	sub_non_syn	c.409G>A	p.D137N
	3	12-1b-E8-3	486	C	T	222	13	promoter	+	mexR_mexA	mexA promoter	sub	Na	Na
No-drug	4	12-1a-F8-20	No mut.	-	-	-	-	-	Na	-	-	-	-	-
Fast-reg.	5	12-1a-D8-18	197	C	T	222	28	CDS	-	cpxS = PA14_22730	two-component sensor (homology to PA3206)	sub_non_syn	c.562C>T	p.G188S
			744	624	CTTGTCGCCAACC TTCGGCG	CCGCCAGC	107	3	CDS	-	cycB	cytochrome c5	indel	c.235_255CTTGT CGCCAACCTTCG GCG>CCGCCAG C
	6	12-1a-C2-13	368	T	C	222	18	CDS	+	parS	two-component sensor	sub_non_syn	c.455T>C	p.V152A
			340	155	GTCATGCCCGGAT	GT	214	13	CDS	-	nalD	TetR family transcriptional regulator	indel	c.461_471del
			114											
			7											

	7	12-1b-F2-3	342 117 8	CGTTCGCACTTGA GGT	C	159	7	CDS	-	mexZ	transcriptional regulator AmrR	indel	c.329_343del	p.YLKCER110_C
			280 099 6	CGCGCCGATCATG	CG	136	5	CDS	+	catB	muconate cycloisomerase I	indel	c.731_741del	p.A244Gfs39
			197 751 9	T	G	86	4	CDS	-	cpxS = PA14_22730	two-component sensor (homology to PA3206)	sub_non syn	c.487T>G	p.T163P
			317 569 5	T	TGGCCGA	55	14	promote r	-	PA14_35700 _PA14_3571 0	PA14_35700 promoter	indel	Na	Na
			359 310 7	CC	CCCCTCACC	43	5	CDS	-	PA14_40260	hypothetical protein	indel	c.2833_2834insC CTCACC	p.G946Efs376
		12-1b-F2-4	280 099 6	CGCGCCGATCATG	CG	214	17	CDS	+	catB	muconate cycloisomerase I	indel	c.731_741del	p.A244Gfs39
			856 314	AGCGTCACGCTGG	AG	183	8	CDS	+	PA14_09960	hypothetical protein	indel	c.375_385del	p.S125Rfs53
			197 751 9	T	G	125	5	CDS	-	cpxS = PA14_22730	two-component sensor (homology to PA3206)	sub_non syn	c.487T>G	p.T163P
Slow-reg.	8	12-1a-B8-15	123 684 1	T	C	222	14	CDS	-	pepA	leucyl aminopeptidase	sub_syn	c.6T>C	p.E2E
			368 334 2	A	C	222	16	CDS	+	parS	two-component sensor	sub_non syn	c.391A>C	p.T131P
			289 249 8	C	CA	78	9	CDS	+	gcvT2	glycine cleavage system protein	indel	c.589insA	p.Y197Ifs232
			370 748 4	G	GGTGCTGA	41	10	CDS	+	nirB	assimilatory nitrite reductase large subunit	indel	c.1438_1439insG TGCTGA	p.V480Gfs359
		12-1a-B8-18	368 336 1	T	C	222	13	CDS	+	parS	two-component sensor	sub_non syn	c.410T>C	p.L137P
			341 831 6	GTAGCCCTCGGCG CT	G	123	5	CDS	-	galU	UTP-glucose-1-phosphate uridylyltransferase	indel	c.769_782del	p.S257Hfs96

		341 832 9	C	CCTCGAT	41	2	CDS	-	galU	UTP-glucose-1-phosphate uridylyltransferase	indel	c.769_770insCTC GAT	p.S257NRG
	48-1a- B8- *15	563 732 0	G	A	222	15	CDS	+	pmrB	two-component sensor	sub_non syn	c.637G>A	p.E213K
		155 122 1	CACGAA	CA	214	23	CDS	-	nalD	TetR family transcriptional regulator	indel	c.400_403del	p.F134Cfs25
	48-1a- B8- *18	155 106 4	GATCGAACAGGC	G	214	22	CDS	-	nalD	TetR family transcriptional regulator	indel	c.547_557del	p.R182Pfs61
	48-1a- B8- *20	123 684 1	T	C	177	7	CDS	-	pepA	leucyl aminopeptidase	sub_syn	c.6T>C	p.E2E
		368 334 2	A	C	165	8	CDS	+	parS	two-component sensor	sub_non syn	c.391A>C	p.T131P
9	12-1b- A8-2	486 467	CGGG	CGGGGG	214	16	CDS	-	mexR	multidrug resistance operon repressor	indel	c.34insG	p.A12Gfs106
		855 966	C	A	179	9	CDS	+	PA14_09960	hypothetical protein	sub_non syn	c.25C>A	p.R9S
		317 569 5	T	TGGCCGA	68	20	promote r	-	PA14_35700 _PA14_3571 0	PA14_35700 promoter	indel	Na	Na
	12-1b- A8-4	855 966	C	A	222	19	CDS	+	PA14_09960	hypothetical protein	sub_non syn	c.25C>A	p.R9S
	12-1b- A8-7	394 814	C	T	222	12	CDS	+	ptsP	phosphoenolpyruvate phosphotransferase	sub_non syn	c.2045C>T	p.P682L
		155 114 6	GGTCATGCCCGGA TG	GG	214	18	CDS	-	nalD	TetR family transcriptional regulator	indel	c.460_472del	p.H154Pfs12
		317 569 5	T	TGGCCGA	74	15	promote r	-	PA14_35700 _PA14_3571 0	PA14_35700 promoter	indel	Na	Na
		535 850 8	CG	C	51	17	CDS	+	PA14_60140	xerD-like integrase	indel	c.770delG	p.R257Lfs172
	12-1b- A8-9	855 966	C	A	217	9	CDS	+	PA14_09960	hypothetical protein	sub_non syn	c.25C>A	p.R9S

			411 666 9	G	T	41	11	CDS	-	PA14_46270	helicase	sub_non syn	c.358G>T	p.L120R
			411 666 8	A	C	40	11	CDS	-	PA14_46270	helicase	sub_non syn	c.359A>C	
	10	12-1b- A2-4	563 770 2	A	C	222	11	CDS	+	pmrB	two-component sensor	sub_non syn	c.1019A>C	p.H340P
			486 113	T	G	221	9	CDS	-	mexR	multidrug resistance operon repressor	sub_non syn	c.388T>G	p.T130P
			317 569 5	T	TGGCCGA	52	15	promote r	-	PA14_35700 _PA14_3571 0	PA14_35700 promoter	indel	Na	Na
		12-1b- A2-8	486 113	T	G	222	14	CDS	-	mexR	multidrug resistance operon repressor	sub_non syn	c.388T>G	p.T130P
			390 652 1	CCCCGACGAGGCT G	C	214	15	CDS	-	PA14_43870	hypothetical protein	indel	c.786_798del	p.S262Rfs28
			289 249 8	C	CA	76	10	CDS	+	gcvT2	glycine cleavage system protein	indel	c.589insA	p.Y197Ifs232
			407 965 1	G	C	46	14	CDS	-	PA14_45890	RND efflux transporter (homology to PA1436, muxB)	sub_syn	c.612G>C	p.T204T
Rando m	11	12-1b- C8-*3	368 408 9	G	A	217	9	CDS	+	parS	two-component sensor	sub_non syn	c.1138G>A	p.D380N
		12-1b- C8-*4	123 596 4	TGGCGGCG	TGGCG	214	14	CDS	-	pepA	leucyl aminopeptidase	indel	c.878_880del	p.A294del
			368 408 9	G	A	110	4	CDS	+	parS	two-component sensor	sub_non syn	c.1138G>A	p.D380N
	12	12-1a- E2-4	999 954	A	C	222	14	CDS	-	ispA	Geranyltranstransferase (isoprenyl biosynthesis)	sub_non syn	c.745A>C	p.Y249D
			289 249 8	C	CA	111	11	CDS	+	gcvT2	glycine cleavage system protein	indel	c.589insA	p.Y197Ifs232

13	12-1b-G2-16	155	T	A	222	11	CDS	-	nalD	TetR family transcriptional regulator	sub_non syn	c.94T>A	p.S32C
		152 6											
		259 205 0	AT	A	214	16	CDS	-	nuoG	NADH dehydrogenase subunit G	indel	c.2134delT	p.M712Cfs71
14	12-1a-D2-3	467 880 7	CGGTCAGTTGCGG A	C	165	8	CDS	-	aotJ	arginine/ornithine binding protein	indel	c.602_618del	p.P203Mfs76
		197 775 8	CTTTCGT	C	45	2	CDS	-	cpxS = PA14_22730	two-component sensor (homology to PA3206)	indel	c.248CTTTCGT> C	p.S83Na
	12-1a-D2-6	342 111 9	GTT	GT	152	8	CDS	-	mexZ	transcriptional regulator AmrR	indel	c.402delT	p.Q134Hfs105
		197 775 8	CTTTCGT	C	64	3	CDS	-	cpxS = PA14_22730	two-component sensor (homology to PA3206)	indel	c.248CTTTCGT> C	p.S83Na
	12-1a-D2-7	197 775 8	CTTTCGT	C	110	4	CDS	-	cpxS = PA14_22730	two-component sensor (homology to PA3206)	indel	c.242_247del	p.S83Na
15	12-1b-H2-17	436 987 3	A	C	222	10	CDS	-	phoQ	two-component sensor	sub_non syn	c.779A>C	p.V260G
		588 874 2	GCGTC	GCGTCGTC	214	16	CDS	+	PA14_66100	hypothetical protein	indel	c.1040_1041insG TC	p.347_348insS
	12-1b-H2-18	436 987 3	A	C	222	17	CDS	-	phoQ	two-component sensor	sub_non syn	c.779A>C	p.V260G
		393 300 7	AGGCGGCAACGGC GGCAACGGCGGCA ACGGCGGCAACGG CGGCA	AGGCGGCAACGGC GGCAACGGCGGCA ACGGCGGCAACGG CGGCAACGGCGGC A	214	9	CDS	+	PA14_44190	sugar MFS transporter	indel	c.736_737insAC GGCGGCA	p.245_246insT AA
		393 302 8	CGGCAACGG	CGGCAACGGTGGC AACGG	214	9	CDS	+	PA14_44190	sugar MFS transporter	indel	c.723_724insTG GCAACGG	p.241_242insG GN
		589 344 5	GGCGGTCGAGCG	GGCG	193	7	CDS	-	wapH	glycosyl transferase family protein	indel	c.1002_1009del	p.L334Pfs340

	16	12-1a-H8-16	550 556 8	TGGGCCAGGGCCA G	TGGGCCAGGGCCA GGGCCAG	193	8	CDS	-	hemA	glutamyl-tRNA reductase (tetrapyrrole biosynthesis)	indel	c.1215_1216insG GCCAG	p.409_410LA
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*Genetic changes compared to *Pseudomonas aeruginosa* PA14 *wt* strain as determined by whole-genome resequencing (Illumina MiSeq2x150bp PE, Nextera libraries). Intergenic mutations are listed if they affected promoter regions, which were identified using PePPER. Isolates are coded with AA-BB-CC-DD: AA, transfer; BB, plate; CC, well; DD, colony. SEQ, sequence of antibiotics from evolution experiment; POS, position in genome; REF, allele in reference genome; ALT, alternative allele; QUAL, quality score; ST, strand; CDS, coding sequence; Na, not applicable.

Table S2. Growth rate as determined from CFU-counts confirmed OD-based measurements.

Phenotype	Strain	Doubling time CFUs (min)	Fitness cfu %	Relative growth rate OD ₆₀₀
Ancestor	PA14	41.5	100	1
Tolerance	12-1a-E2-4	56.3	73	0.593
CIP ^R	12-1a-G8-3	42.1	98	0.977
GEN ^R	12-1b-B2-8	49.4	84	0.976
CAR ^R	12-1b-E8-3	39.9	104	1.02

A superscript ^R denotes resistance against the respective antibiotic. CIP, ciprofloxacin; GEN, gentamicin; CAR, carbenicillin.

Table S3. Minimum duration for killing of tolerant isolate 12-1a-E2-4 (*ispA* Y249D).

Strain	CIP (50 ng/ml)		CAR (100 µg/ml)		GEN (500 ng/ml)		GEN 500 ng/ml (pre-treated with CAR)	
	MDK ₉₀	MDK ₉₉	MDK ₉₀	MDK ₉₉	MDK ₉₀	MDK ₉₉	MDK ₉₀	MDK ₉₉
<i>wt</i>	1.23	2.81	5.37	> 6	2.69	3.46	2.05	2.65
<i>ispA</i>	2.62	> 6	5.85	> 6	> 6	> 6	> 6	> 6

Time in hours to kill 90% (MDK₉₀) or 99% (MDK₉₉) of the starting cell population.

Statistics file

Statistics sheet 1. Evolutionary dynamics. Results of post-hoc pairwise comparisons of main evolution treatments*.

Comparison	Estimate	Std. error	z	p
Early phase (transfers 1-12)				
mono-fast regular	1.13413	0.10973	10.335	<2e-16
mono-slow regular	0.18123	0.08992	2.016	0.0438
mono-random	1.74299	0.16655	10.465	<2e-16
fast regular-slow regular	-0.9529	0.1122	-8.493	<2e-16
fast regular-random	-0.52527	0.21391	-2.456	0.0169
slow regular-random	1.38053	0.173	7.98	2.33E-15
Middle phase (transfers 13-48)				
mono-fast regular	0.3245	0.11259	2.882	0.007901
mono-slow regular	0.06362	0.09168	0.694	0.58529
mono-random	0.66692	0.17155	3.888	0.000607
fast regular-slow regular	-0.26088	0.11437	-2.281	0.033823
fast regular-random	0.01792	0.21932	0.082	0.934879
slow regular-random	0.53968	0.17619	3.063	0.006574
Late phase (transfers 49-96)				
mono-fast regular	0.37051	0.2488	1.489	0.2729
mono-slow regular	0.03939	0.08272	0.476	0.7607
mono-random	1.07341	0.41217	2.604	0.0552
fast regular-slow regular	-0.29173	0.25141	-1.16	0.3688
fast regular-random	0.09809	0.41839	0.234	0.8146
slow regular-random	0.87646	0.42195	2.077	0.1134

* Post-hoc pairwise comparisons based on z statistics, following analysis of a general linear mixed model (GLMM), including relative biomass (relative AUC) as the response variable and sequence and transfer as fixed factors and starting culture and replicate population as nested random factors. All significant p values are shown in bold (p values were corrected for multiple testing using *false discovery rate*).

Statistics sheet 2. Extinction events. Fishers's exact tests*.

Main treatment type	Extinct	Growth		
Fast - Slow				
fast regular	11	25	p = 0.01201	<i>odds-ratio</i> = 7.287493
slow regular	2	34		CI95 = 1.407, 73.282
Random - Fast				
random	11	25	p = 0.15	<i>odds-ratio</i> = 1.9834
fast regular	13	59		CI95 = 0.70135, 5.564
Random - Slow				
random	2	34	p = 0.137	<i>odds-ratio</i> = 0.2696
slow regular	13	59		CI95 = 0.0279, 1.3024

*All significant p values are shown in bold.

Statistics sheet 3. Diversity. Results of post-hoc pairwise comparisons of main evolution treatments*.

Comparison	Estimate	Std. error	<i>z</i>	<i>p</i>
Early phase (transfers 1-12)				
mono - fast regular	0.10791	0.15178	0.711	0.596391
nodrug - fast regular	-0.05418	0.21465	-0.252	0.80072
random - fast regular	0.16393	0.13144	1.247	0.424684
slow regular - fast regular	0.79392	0.15178	5.231	1.69E-06
nodrug - mono	-0.16209	0.21465	-0.755	0.596391
random - mono	0.05602	0.13144	0.426	0.744392
slow regular - mono	0.68601	0.15178	4.52	2.06E-05
random - nodrug	0.21811	0.20078	1.086	0.462246
slow regular - nodrug	0.8481	0.21465	3.951	0.000194
slow regular - random	0.62999	0.13144	4.793	8.22E-06

* Post-hoc pairwise comparisons based on *z* statistics, following analysis of a general linear mixed model (GLMM), including within population diversity (Shannon entropy) as the response variable and sequence as fixed factor and transfer as nested random factor. All significant *p* values are shown in bold (*p* values were corrected for multiple testing using *false discovery rate*).

Statistics sheet 4. Repeatability of resistance measurements. Pearson's product-moment correlation.

$t = 30.818$, $df = 118$, $p\text{-value} < 2.2E-16$

CI95 = 0.9192860, 0.9600694

correlation coefficient = 0.943125

coefficient of determination $R^2 = 0.889485$

Formula linear model: $y = f(x) = 0.96063x + 0.01916$

Statistics sheet 5. Multidrug resistance. Results of post-hoc pairwise comparisons of main evolution treatments*.

Comparison	Estimate	Std. error	<i>z</i>	<i>p</i>
Early phase (after transfer 12)				
fast regular-slow regular	-2.0795	0.8207	-2.534	0.0338
fast regular-random	-1.3771	1.4462	-0.952	0.341
slow regular-random	2.7818	1.3965	1.992	0.0696
Middle phase (after transfer 48)				
fast regular-slow regular	-2.45	1.104	-2.219	0.0795
fast regular-random	-1.606	1.913	-0.84	0.4012
slow regular-random	3.294	1.913	1.722	0.1275

* Post-hoc pairwise comparisons based on *z* statistics, following analysis of a general linear mixed model (GLMM), including multidrug resistance (MDR) as the response variable and sequence as fixed factor and population as nested random factor. All significant *p* values are shown in bold (*p* values were corrected for multiple testing using *false discovery rate*).

Statistics sheet 6. Zero resistance sequence 12. Results of post-hoc pairwise comparisons of main evolution treatments*.

Comparison	Estimate	Std. error	<i>z</i>	<i>p</i>
Early phase (after transfer 12)				
sequence 12 - ancestor	0.03068	0.22932	0.134	0.894

* Post-hoc pairwise comparisons based on *z* statistics, following analysis of a general linear mixed model (GLMM), including relative resistance as the response variable and treatment and antibiotic as fixed factor and population as nested random factor. All significant *p* values are shown in bold.

Statistics sheet 7. Cumulative hysteresis influence correlates with MDR. Spearman's rank correlation.

$S = 42$, p -value = 0.0007719

$\rho = 0.8531469$

coefficient of determination $R^2 = 0.7036527$ (calculated via Pearson correlation)

Statistics sheet 8. Growth rate. Dunnett test exponential growth rate*.

Comparison	Estimate	Std. error	<i>z</i>	<i>p</i>
Early phase (after transfer 12)				
fast regular - ancestor	-0.05016	0.051515	-0.974	0.413
mono - ancestor	-0.0055	0.051515	-0.107	0.457
nodrug - ancestor	-0.02229	0.064992	-0.343	0.457
random - ancestor	-0.07863	0.047677	-1.649	0.248
slow regular - ancestor	-0.02872	0.050807	-0.565	0.457
Middle phase (after transfer 48)				
fast regular - ancestor	0.002714	0.016832	0.161	0.61632
mono - ancestor	-0.02479	0.015288	-1.621	0.13121
nodrug - ancestor	0.007714	0.026076	0.296	0.61632
random - ancestor	-0.04116	0.012624	-3.261	0.00278
slow regular - ancestor	-0.01089	0.01202	-0.906	0.30429

* Post-hoc pairwise comparisons based on *z* statistics, following analysis of a general linear mixed model (GLMM), including mean exponential growth rate (*k*) as the response variable and main treatment type as fixed factor and sequence as nested random factor. All significant *p* values are shown in bold (*p* values were corrected for multiple testing using *false discovery rate*).

Statistics sheet 9. Genes overrepresented among mutated genes after transfer 12. Fishers's exact tests*.

Gene	Present	Absent		
cpxS				
fast regular	3	1	$p = 0.02479$	<i>odds-ratio</i> = 16.67657
other	3	20		CI95 = 1.004, 1089.336
mexR				
slow regular	3	5	$p = 0.01915$	<i>odds-ratio</i> = Inf
other	0	19		CI95 = 1.137, Inf

*All significant p values are shown in bold.

Statistics sheet 10. Re-play evolution experiment (Lewontin). Results of post-hoc pairwise comparisons of main evolution treatments*.

Comparison	Estimate	Std. error	z	p
Early phase (after transfer 12)				
12rev - 12 \leq 0	1.4609	0.5946	2.457	0.014
13 - 13rev \geq 0	-0.3286	0.8258	-0.398	0.655

* Post-hoc pairwise comparisons based on z statistics, following analysis of a general linear mixed model (GLMM), including fold-change IC_{75} as the response variable and sequence as fixed factor and antibiotic as nested random factor. All significant p values are shown in bold.

Chapter 4

Short-Form Paper prepared for *Antimicrobial Agents Chemotherapy*

Sequential treatment with three β -lactams in *Pseudomonas aeruginosa* and the evolution of resistance

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Running title

Sequential treatment with three β -lactams

Abstract

Multidrug treatments limit the emergence of resistance. Typically, these treatments involve drugs with distinct targets. We here investigate sequential therapy with three β -lactams and thus antibiotics with the same mechanism of action. Surprisingly, the tested sequential treatments produce high efficacy towards the pathogen *Pseudomonas aeruginosa*, possibly due to conflicting resistance mechanisms against the three drugs. Our findings point to novel treatment options.

Main text

Antibiotic resistance is a global challenge for chemotherapy. Resistance frequently evolves within patients in response to treatment. The emergence of resistance may be delayed by multidrug treatments, which limit the amount of beneficial mutations available for pathogen adaptation and in the case of sequential application can provide additional evolutionary constraints due to collateral sensitivity (1–4) and/or negative physiological responses induced by specific antibiotics (5, 6). Conventionally, multidrug treatments would avoid antibiotic from similar classes, with the rationale of limiting the overlap in the respective sets of resistance mutations, and thus the ensuing cross-resistance. Yet, the dire need for new therapy has elicited a re-consideration of previously avoided treatment options. Dual therapy with antibiotics targeting cell-wall synthesis is now being considered (7), and a first trial indicates improved treatment of methicillin-resistant *S. aureus* (MRSA) (8). We here investigated *in-vitro* the evolutionary adaptive response of *Pseudomonas aeruginosa* to triple β -lactam sequential therapy, using serial transfer evolutionary experiments (Fig. 1).

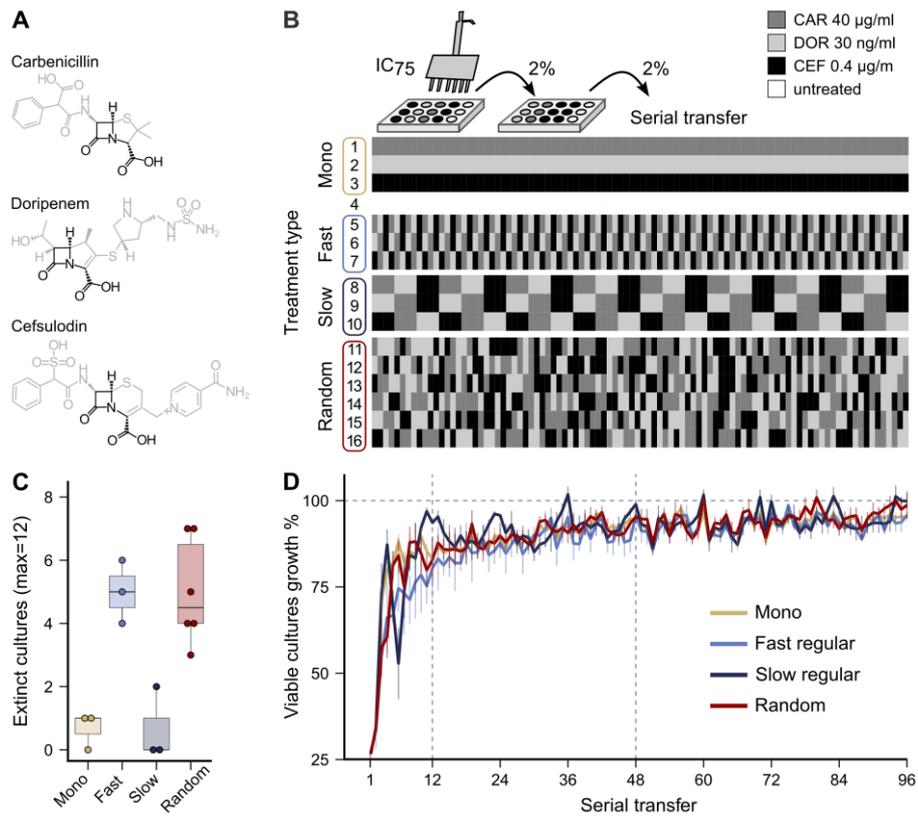


Figure 1. Evolutionary response to triple sequential therapy with three β -lactams in *Pseudomonas aeruginosa*. (A) Chemical structure of the antibiotics with the shared β -lactam structure highlighted in dark. (B) Experimental setup with 16 different treatment protocols belonging to 4 general treatment types. Each protocol is evaluated with 12 replicates and drugs are dosed below MIC, at inhibitory concentration of 75% (IC75). CAR, carbenicillin; CEF, cefsulodin; DOR, doripenem. (C) Treatment type determines treatment efficacy, as indicated by significantly higher extinction frequencies in fast-switching protocols. (D) Evolutionary growth improvements of surviving cultures during treatment (mean \pm CI95; 3-6 sequences per treatment type and 12 biological replicates per sequence; extinct lineages excluded).

Replicate cultures of *P. aeruginosa* PA14 (total = 188, derived from 6 colonies) were treated with low dose of bactericidal antibiotics, reducing growth to 25% of untreated controls (IC₇₅, inhibitory concentration 75). Every 12h 2% of the cultures were transferred to fresh medium, containing either the same or a new antibiotic. The cultures were grown in 96-well plates, incubated in plate readers at 37°C and shaking (BioTek Instruments, USA; Ref. EON), with kinetic measurement of optical density at 600 nm (OD₆₀₀) every 15 min for a total of 96 transfers. In this way we investigated the dynamics of resistance emergence in response to 16 different treatments, belonging to four major treatment types: monotherapy, fast-regular, slow-regular and random therapy (Fig. 1). The experiment precisely followed a setup that we previously used to test sequential treatments with antibiotics from different classes (6), yet in this experiment we used three β -lactams that individually inhibit the DD-transpeptidase activity in cell-wall synthesis (9): carbenicillin (CAR), doripenem (DOR) and cefsulodin (CEF). The antibiotics have the same core structure, but differ in their side chains (Fig. 1A), which produces different susceptibilities to degradation by the chromosomal AmpC β -lactamase of *P. aeruginosa*: AmpC cannot hydrolyze carbapenems like DOR, has low activity against cephalosporins like CEF, yet high activity against CAR (10).

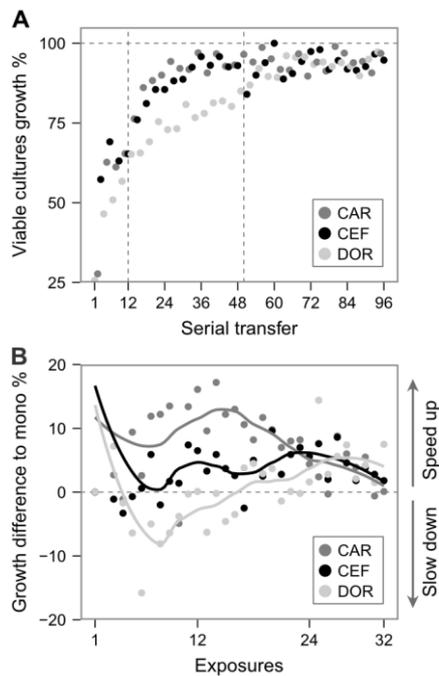


Figure 2. Growth dynamics in fast sequential protocols.

(A) Evolutionary growth improvements for fast protocol #6 during treatment, in more detail. Means of the 7 surviving cultures. Relative growth increases to the antibiotics at different rates. This demonstrates the consecutive evolution of resistance, and thus coexistence of genetic subpopulations. The resulting clonal interference may explain the drop in growth on CEF around transfer 50, and the subsequent oscillations in CEF^R. (B) Mean difference of growth during exposures to particular antibiotics in fast sequential protocols #5-7, compared to growth in monotherapies after the same number of exposures to that drug. X-axis denotes exposures to particular antibiotic, and thus goes to 96/3 = 32. Triple exposure accelerates adaptation compared to monotherapy against CAR and CEF, but slows down adaptation against DOR.

Experimental cultures showed dichotomy in treatment response, with experimental lineages either going extinct (26%, 49/188) or evolving resistance against β -lactam treatment (139/188, 74%). Extinction occurred significantly more often in fast and random protocols (average 42%) compared to monotherapy or slow protocols (6%, Fig. 1C, Fisher's exact tests, $p < 0.0003$). Intriguingly, the total percentage of dead cultures was 2-fold that observed in the previous experiment (27/190, 14%) (6), indicating a treatment advantage of converging on a single mechanism of action. Cultures that survived treatment rapidly evolved resistance against β -lactam treatment, following saturation dynamics. Within the first 12 transfers fast-regular protocols delayed increases in growth compared to all other treatment types (General linear mixed model, pairwise post-hoc tests, $z > 2.46$, $p < 0.03$, Supplementary Table 1), but thereafter there were no significant differences among main treatment types (Fig. 1D). We conclude that fast sequential treatments with three β -lactams increase extinction, but only mildly decelerate resistance evolution.

The increased extinction in fast sequential treatments can theoretically be caused by two different processes: drug-induced negative physiological responses, or, alternatively, antagonistic pleiotropy of resistance mutations (collateral sensitivity within β -lactams). The data presented here, indicate that it is the latter. Physiological responses can be excluded in this context, because all three drugs are known to elicit a similar response, namely induction of AmpC (10). Likewise priming experiments showed no influence on killing rates (data not shown). A first line of evidence for the importance of collateral sensitivity is the requirement of several mutations for the emergence of triple resistance, demonstrated by the consecutive adaptation to the component drugs during triple exposure (Fig. 2A). Emergence of triple resistance in fast protocols occurs by sequential acquisition of resistance, first against CAR/CEF (i.e., CAR^R/CEF^R) and later against DOR (i.e., DOR^R; Fig. 2A). The signature for antagonistic pleiotropy is slower growth improvement against particular antibiotics in fast-sequential compared to monotherapy, i.e. lower growth after a certain number of exposures to a particular antibiotic. This analysis reveals that triple sequential exposure accelerates adaptation against the antibiotics CAR and CEF, but delays the emergence of

DOR^R (Fig. 2B), indicating a genetic trade-off. The trade-off is supported by previous work on DOR^R, which is most commonly achieved by loss-of-function mutations in the porin OprD that catalyzes uptake of carbapenems (11). DOR appears to select on a narrow mutation space: In a previous experiment, all replicate populations treated with DOR produced resistance substitutions in OprD, and no other genes (3). The expression of OprD-mediated DOR^R depends on the activity of AmpC (12), which is often altered by mutations in penicillin binding proteins (13, 14) that confer CAR^R and CEF^R (3). Conversely, OprD mutations cause collateral-sensitivity against other β -lactams, especially CAR (3), which likely explains the increased extinction during fast sequential treatments. Altogether, the evolution of triple resistance is limited by the requirement of several mutations which involve mostly trade-ups, but also collateral sensitivity and epistasis.

We conclude that triple sequential treatment of *P. aeruginosa* with β -lactams has improved efficacy compared to monotherapy, because it elevates extinction. Treatment efficacy is limited over time, due to the rapid evolution of resistance. An interesting question for future work will be how the evolved resistance mutations affect susceptibility to antibiotics with different mechanisms of action. If the mutations do not increase cross-resistance, then triple therapy is a promising therapeutic option, because it can be applied with high efficacy while at the same time maintaining future treatment options with distinct antibiotic classes.

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Supplementary Table 1. Results of post-hoc pairwise comparisons of main evolution treatments*.

Comparison	z	p
Early phase (transfers 1-12)		
mono-fast regular	2.695	0.0241
mono-slow regular	0.243	0.9049
mono-random	0.119	0.9049
fast regular-slow regular	-2.462	0.0276
fast regular-random	-2.650	0.0241
slow regular-random	-0.130	0.9049
Middle phase (transfers 13-48)		
mono-fast regular	1.039	0.359
mono-slow regular	-1.242	0.359
mono-random	-1.095	0.359
fast regular-slow regular	-2.088	0.137
fast regular-random	-1.999	0.137
slow regular-random	0.189	0.85
Late phase (transfers 49-96)		
mono-fast regular	0.051	0.96
mono-slow regular	-0.665	0.641
mono-random	-1.710	0.389
fast regular-slow regular	-0.622	0.641
fast regular-random	-1.515	0.389
slow regular-random	-0.980	0.641

* Post-hoc pairwise comparisons based on z statistics, following analysis of a general linear mixed model (GLMM), including relative growth as the response variable and sequence and transfer as fixed factors and starting culture and replicate population as nested random factors. The defined model provided a better fit than a minimal model for all three phases (*Likelihood ratio* > 168, $p < 0.001$). All significant p values are shown in bold (p values were corrected for multiple testing using *false discovery rate*).

General discussion

The discovery of antibiotics in the last century revolutionized medicine and greatly reduced the fear of bacterial infections. However, the efficacy of antibiotics is inseparably linked to the selection of antibiotic resistance. The irrational use of antibiotics has thus culminated in a global pool of evolved resistant bacteria that are now considered the most pressing medical challenge of the century (Laxminarayan, 2014; Bundesministerium für Gesundheit, 2017). The antibiotic crisis is fueled by evolution, and thus by considering principles from evolutionary ecology we may be able to use antibiotics in new ways that inhibit the emergence of resistance (Baym, Stone, & Kishony, 2016). Sequential treatments – which I investigated in this dissertation – hold hope, as they can dynamically respond to bacterial adaptation or altogether divert adaptive priority away from resistance.

Summary of investigations

At the start of the project, we searched the literature for principles from evolutionary ecology that could limit resistance evolution, when applied in therapy (Chapter 1). We discovered a wealth of published data that had disappeared from common knowledge, and were not easily accessible because of changes in terminology. The result of our survey is that an integration of temporal variation in antibiotic therapy, i.e. sequential treatment protocols, has potential to limit resistance evolution. Sequential multidrug treatments may delay resistance emergence compared to combination and monotherapy because of selection dynamics that arise from temporal contrasts:

- i) Fluctuating selection increases clonal interference
- ii) Change of antibiotics can potentiate treatment due to cross-stress sensitivity
- iii) Change of antibiotics select for re-sensitization due to collateral sensitivity
- iv) Unpredictable changes limit fitness benefits from genetic and physiological correlations

Most of the proposed ideas had not been tested as for their applicability in therapy or with sufficient sample size to reach statistically verifiable conclusions. The aspect that had received recent attention, was collateral sensitivity. It was argued in the literature that alternations of drugs with reciprocal collateral sensitivity serially re-sensitized bacteria to treatment (Imamovic & Sommer, 2013; Pál, Papp, & Lázár, 2015). We tested the evolutionary stability and thus the potential applicability of collateral sensitivity cycling in a relevant pathogen (Chapter 2). We discovered that, although collateral sensitivity generally constrained adaptation, *P. aeruginosa* could escape the re-sensitization cycle with varying degrees of difficulty. The stability of reciprocal collateral sensitivity was limited by switching order, and the total number of switches.

We next tested how the other predicted parameters (switching rate, cross-stress sensitivity, and unpredictability) affected the dynamics of resistance evolution. To this end, we performed a small series of five evolution experiments, of which two have been analyzed and are presented as part of this dissertation. These two experiments differed in the applied antibiotics, which either had the same (Chapter 4) or different mechanisms of action (Chapter 3). Our overall results showed that temporal variation could slow down adaptation, however this result could not be generalized for all fluctuating treatments but instead depended on the specified sequence characteristics and antibiotics.

The experiments confirmed the anticipated high potential of *P. aeruginosa* for resistance evolution. Several populations evolved a multidrug resistant phenotype within 60 generations (12 transfers, 6 days). The rapid adaptation occurred significantly more often in slow antibiotic cycling, which is therefore clearly sub-optimal, as additionally indicated by low extinction frequencies which were not significantly different from monotherapies.

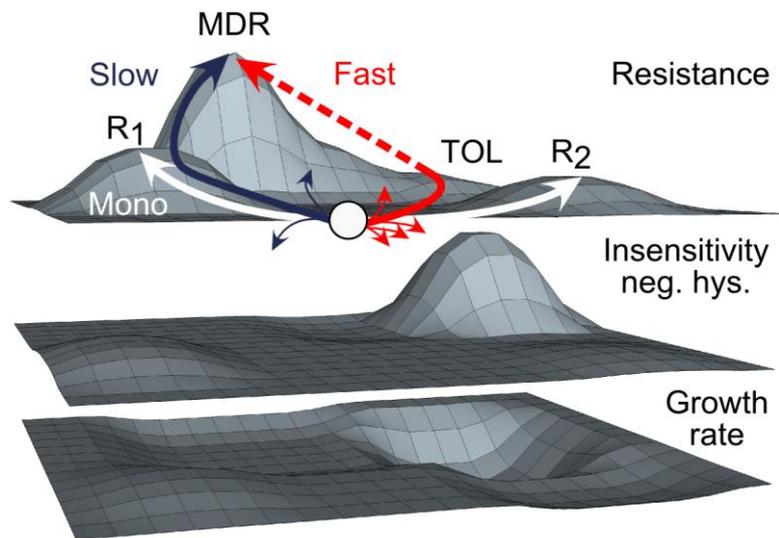


Figure 1. Fitness landscape in drug environments. Fitness landscapes are a simplified illustration of evolution originally introduced by Sewall Wright, 1932. Fitness (height) is a function of the genotype space (surface) and adaptation is the probabilistic movement on this surface as consequence of selection (slope). The superimposed arrows denote the most probable mutation trajectory (adaptive path) for a given antibiotic treatment strategy, that typically proceeds along the steepest slope (strongest selection). Short arrows indicate extinction events. In drug environments, a large amount of fitness is resistance (top layer). Antibiotic hysteresis adds a second fitness dimension. The low resistance gained during fast switching treatment can now be explained by the hill-climbing on the second layer, where fitness increases by evolving insensitivity to negative hysteresis. In reality, fitness landscapes are n -dimensional. The most important additional layer in this context is physiological growth rate (bottom layer). MDR, multidrug resistance; neg. hys., negative hysteresis; R₁, R₂, resistance against different antibiotics; TOL, antibiotic tolerance.

Fast sequential treatments, however, had improved efficacy, with significantly increased extinction rates and slower resistance increases. There was variation according to drug order, which then was explained by physiological cross-stress interactions induced by particular antibiotics, which we call *antibiotic hysteresis*. Antibiotic hysteresis either protected cells from antibiotics (positive hysteresis), potentially buffering collateral sensitivity, or potentiated treatment (negative hysteresis). A potential mechanism for positive hysteresis is antibiotic-specific induction of efflux pumps (Morita, Tomida, & Kawamura, 2014). Negative hysteresis occurs between many β -lactam and aminoglycoside antibiotics (recent experiments, data not shown), and is explained by β -lactam induced acceleration of aminoglycoside uptake (Plotz & Davis, 1962).

Hysteresis density in the sequential treatment protocols explained the contrasting adaptive responses to fast and slow antibiotic switching. The differences were caused by different adaptive priorities in two regimens and a genetic trade-off. The treatment protocols with high densities of negative hysteresis selected towards insensitivity to negative hysteresis, which was ultimately associated with low resistance or even tolerance. Conversely, evolved genotypes from slow switching treatments had multidrug resistance, but maintained sensitivity to negative hysteresis. Our findings thereby added a new dimension to fitness landscapes in drug environments (Figure 1), and the ensuing competition of adaptive priorities between the fitness dimensions explained the most probable evolutionary outcome. Altogether, this indicated that the probabilistic evolutionary outcome can be modulated by changing hysteresis density. We tested this prediction with sequences of identical drug proportions and equal number of switches, but slightly different levels of negative hysteresis. Hysteresis effects were direction dependent, and the experimental test was performed by reversing two drug protocols and comparing their treatment efficacies. The obtained data confirmed that changes in hysteresis predictably altered treatment success and evolutionary dynamics. Altogether, negative hysteresis was a potent adaptive constraint for resistance evolution in our experiment, and could thus potentially be applied to limit resistance emergence during antibiotic therapy.

In Chapter 4 we the complementary evolutionary dynamics to the experiments in Chapter 3, using sequential treatments with three β -lactam antibiotics. These drugs did not display negative hysteresis, but rather individually upregulated the expression of an intrinsic resistance determinant, the AmpC β -lactamase. Resistance evolution occurred at similar rates in fast, slow and random protocols. Surprisingly, we observed increased extinction rates in fast switching protocols. Indeed, extinction was generally increased 2-fold compared to the previous experiment in Chapter 3. The effect was explained by emergence of a strong genetic trade-off, in the most common resistance mechanism to one of the antibiotics, doripenem. Multidrug treatments with convergence on one mechanism of action may thus be an underappreciated treatment option.

Altogether, our results indicate several ways to potentially increase the sustainability of antibiotic therapy, which are illustrated in Figure 2.

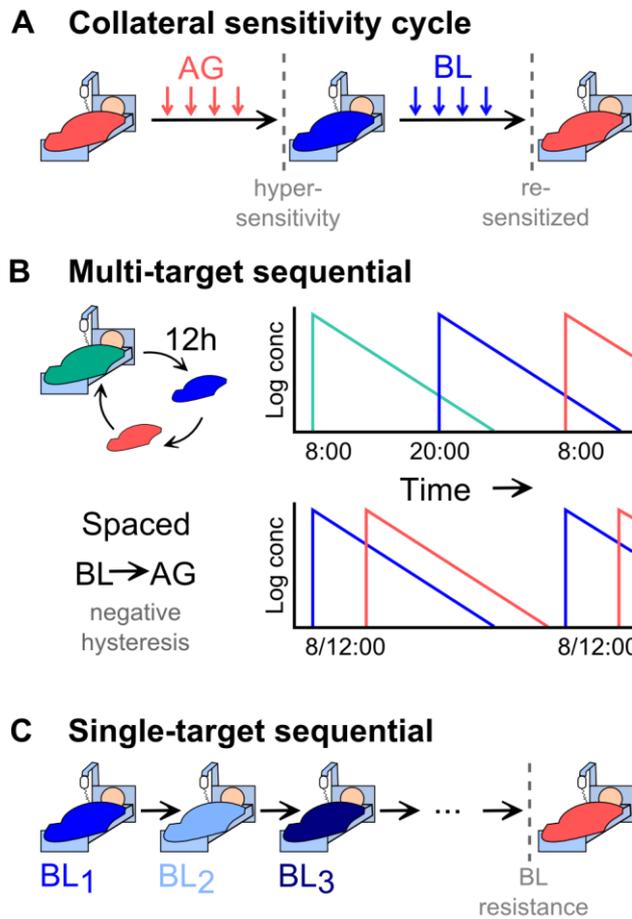


Figure 2. Treatments that impede emergence of multidrug resistance. (A) Collateral sensitivity cycling works for a single cycle, when treatment starts with the appropriate antibiotic. Patients receive several serial doses of an antibiotic, and treatment is then changed to exploit collateral sensitivity of the most common resistance mechanisms. The inversion selects for re-sensitization to the first antibiotic, which should be applied for a single dose only. AG, aminoglycoside; BL, β -lactam. (B) Sequential therapy with fast switching and antibiotics that have different mechanisms of action. Treatment efficacy is improved by antibiotic hysteresis. Two dosing regimens are recommended, serial or spaced administration. Serial administration uses three drugs, similar to the performed evolution experiments, with the risk of selecting multidrug resistant strains in the long-term. Spaced administration emphasizes negative hysteresis and keeps the third drug as backup. (C) Sequential therapy with three β -lactams aims at increased extinction. An emergence of β -lactam resistance can be countered by switching to antibiotics of a different class.

Applicability of sequential drug treatments

How applicable are the postulated treatment strategies? Their clinical success is contingent on a variety of additional biological factors *in vivo*. These factors include, among others, differences in the environment, growth behavior, host factors, and pathogen population diversity. It is increasingly realized that antibiotic susceptibility depends on the physiological and metabolic state of the cell (Hughes & Andersson, 2017; Yang, Bening, & Collins, 2017), which may be altered *in vivo*. Growth in biofilms strongly decreases drug penetration and may thus enable persistence (Stewart, 2002). The adverse side-effects of antibiotics on bodily functions and the disturbance of gut microbiota put pharmacological constraints on multidrug treatments. Biological variation in pathogen isolates from the same patient (Wilder, Allada, & Schuster, 2009; Imamovic *et al.*, 2018) complicates targeted treatment approaches. An ultimate answer as for the applicability of new treatment concepts can therefore only be achieved after thorough tests first in animal models and then in clinical trials. Nevertheless, some important indicators emerge from comparison with the literature.

Collateral sensitivity cycling

Our investigations suggest that the applicability of collateral sensitivity cycling may be limited by alternative evolutionary trajectories. Whilst the most common resistance mechanisms may cause collateral sensitivity, there are rare cost-free mutations (Kim, Lieberman, & Kishony, 2014; Barbosa *et al.*, 2017). Collateral sensitivity cycling may thus restrict the substrate for adaptation, but does not serially re-sensitize pathogen populations, as originally postulated (Imamovic & Sommer, 2013). Our experiments show that the evolutionary stability of collateral sensitivity depends on the starting antibiotic. Aminoglycoside resistant strains were readily re-sensitized by selection with β -lactam, but β -lactam resistant populations maintained their original resistance when exposed to aminoglycosides. The observed history-dependence has important implications for therapy: collateral sensitivity cycling with the tested antibiotics can only be carried out for a single cycle (Figure 2A), which should then be concluded with a single dose of the first antibiotic. The order effect may be less pronounced with other drug pairs. For instance, Yen and Papin recently evaluated cycling with the ciprofloxacin (CIP) and piperacillin (PIP). The authors observed that CIP^R populations of *P. aeruginosa* became re-sensitized by extended PIP selection, and *vice-versa* (Yen & Papin, 2017). CIP and PIP showed asymmetric collateral sensitivity, so that the re-sensitization may actually be explained by activity of other factors, *e.g.* reduced metabolic growth rate.

An additional limitation of collateral sensitivity cycling is the high phenotypic diversity in clinical isolates. Replicate isolates from the same patient show pronounced variation with as for their resistance profiles (Imamovic *et al.*, 2018). Whilst homogenous populations may predictably respond to treatment, it is difficult to anticipate the evolutionary trajectory of such diverse populations. Drug treatments will likely rather sort isolates, than steer evolution into exploitable trajectories. I conclude that the applicability of collateral sensitivity cycling is questionable because of the discussed limitations.

Multi-target sequential therapy

In contrast, there are good indications for the applicability of faster sequential treatments with antibiotics of different classes. Previous work highlights that daily drug alternation can, with certain pairs of antibiotics, slow down the emergence of dual resistance (Perron, Gonzalez, & Buckling, 2007; Kim *et al.*, 2014; Roemhild *et al.*, 2015; Yoshida *et al.*, 2017) and even cause extinction at sub-lethal dosage (Roemhild *et al.*, 2015). Furthermore, sequential treatment showed increased potency in the treatment of biofilms (Rojo-Molinero *et al.*, 2016), which resemble the lifestyle of many pathogens *in vivo*. The mechanistic explanation for the efficacy of fast switching treatments is unclear, although there are indications for drug-class specificity. For example, a fraction of drug pairs from a larger set suppressed the evolution of dual resistance in *E. coli* and all drug pairs that did so, included polymyxin (Yoshida *et al.*, 2017). In alternating treatments of *P. aeruginosa*, exposure to cell-wall-targeting antibiotics slowed down growth increases on antibiotics with different targets (Roemhild *et al.*, 2015). Several authors connected the efficacy of sequential treatments to collateral sensitivity and thus a property of selected genetic variants. Whilst this was a good predictor in a large data set (Yoshida *et al.*, 2017), it was a poor predictor for other data sets (Kim *et al.*, 2014; Roemhild *et al.*, 2015). This variation suggests that additional factors contribute to the efficacy of fast sequential treatments. These may include fitness costs (Andersson & Levin, 1999), and epistasis (Weinreich *et al.*, 2006); but also more immediate post-antibiotic effects (MacKenzie & Gould, 1993; Srimani *et al.*, 2017), and antibiotic hysteresis.

Although collateral sensitivity may generally limit resistance evolution by narrowing down the number of potential adaptive mutations, it does not explain, why fast switching inhibited resistance increases compared to slower switching. A decelerating effect caused by collateral sensitivity should theoretically increase with the frequency of resistant variants and thus interval length. Therefore, maximum inhibition is expected for slow switching. Yoshida *et al.* tested 4 drug pairs with three switching rates. If there were differences by switching rate, then they occurred in the opposite direction: resistance gains were lower for fast compared to slow cycling in two pairs (chloramphenicol/polymyxin, chloramphenicol/nitrofurantoin), but not in the other two (kanamycin/polymyxin, kanamycin/nalidixic acid). The four pairs differed with respect to their collateral sensitivity properties (Yoshida *et al.*, 2017), which did not explain the observed differences. A more probable explanation – in line with my experimental findings in Chapter 3, – is drug-specific antibiotic hysteresis. Antibiotic hysteresis was cryptic in the experimental setup of Yoshida *et al.*, because the authors did not measure growth rate or population size, but only increases of the MIC (minimal inhibitory concentration).

Hysteresis may be an alternative explanation for the observed extinction during alternations of cefsulodin/gentamicin and doripenem/ciprofloxacin in *P. aeruginosa* (Roemhild *et al.*, 2015), as indicated by two lines of evidence: antibiotic hysteresis is common with β -lactams (recent experiments; data not shown); collateral sensitivity is weak in these drug pairs, due to generally small effect size and sign-variation between alternative mutations (Roemhild *et al.*, 2015; Barbosa *et al.*, 2017).

Kim *et al.* observed only mildly increased efficacy of drug alternation compared to monotherapy, along with high overlap in the selected mutation spectra (Kim *et al.*, 2014). The small effect size may be a byproduct of the experimental procedure that excluded hysteresis effects, because bacteria were frozen and thawed at every transfer (Kim *et al.*, 2014). Hysteresis effects are stable for several hours during antibiotic stress (Plotz & Davis, 1962), yet they rapidly disappear when

bacteria are in permissive conditions, typically lasting for ~1 generation (recent experiments, data not shown). Altogether, antibiotic hysteresis may substantially contribute to the decelerating effect of multi-target sequential treatments. To ensure immediate and long-term treatment benefits from antibiotic hysteresis, sequential therapy should avoid gaps during sequential drug exposures (Figure 2B overlapping concentrations).

Intriguingly, the clinical applicability of negative hysteresis has been tested, unknowingly, in the treatment of chronic lung infections with *P. aeruginosa* in cystic fibrosis patients (Guggenbichler *et al.*, 1988). Sequential dosing with a 4h gap (so called *spaced administration*) increased bactericidal effect 10-100x and delayed post-antibiotic regrowth compared to simultaneous dosing (combination treatment). Spaced administration enabled eradication of *P. aeruginosa* from the lungs, sometimes for up to a year, which was not achieved by combination treatments. Patient well-being improved during therapy and the authors did not notice unusual adverse effects (Guggenbichler *et al.*, 1988). The clinical pharmacodynamics had good overlap to preliminary *in vitro* experiments (König *et al.*, 1986). The used antibiotics were very similar and sometimes identical to those applied in the experiments of Chapter 3 and negative hysteresis was recently confirmed for these drugs in our lab (data not shown), indicating that the observed treatment efficacy was caused by antibiotic hysteresis. Nevertheless, additional research into the mechanism, generality, and specificity of hysteresis effects will help to further elucidate the applicability of spaced administration, especially in the treatment of infections by other pathogens.

Single-target sequential therapy

Sequential therapy with several β -lactams is a mostly unexplored treatment option. The strength of this approach is the elevated extinction probability (Chapter 4) that was achieved without selecting for broad multidrug resistance and therefore without the risk of restricting future treatment options. Emerging resistance against β -lactams may be countered by switching treatment (Figure 2) to drugs of a different class. Dual therapy with vancomycin and the β -lactam flucloxacillin has recently been successfully tested in the treatment of methicillin resistant *Staphylococcus aureus* (MRSA), in a multicenter clinical trial (Davis *et al.*, 2016), which has elicited a reconsideration of β -lactam combinations for the treatment of refractory bacteremia (Bartash & Nori, 2017). Although the sequential dosing of several β -lactams has not been tested clinically, it may be comparatively easily implemented. The approach does not rely on antibiotic hysteresis, and therefore treatments do not need to ensure overlapping concentration windows.

Outlook

Solid *in vivo* data is required to justify the application of novel treatment strategies. Treatments with immediate benefits may altogether be more applicable because they improve treatment efficacy without the requirement for prior resistance emergence. Fast switching treatments with antibiotics of different classes may have potential for clinical application, because they are supported by independent *in vitro* studies. Their success in comparison to more slowly switching treatments, may largely be explained by antibiotic hysteresis, although this has thus far been concealed by experimental setups. It is important to recall that resistance increases were only inhibited with particular antibiotic pairs. Alternations of other drug pairs had only minor effects on adaptation rates and dual resistance evolved quickly (Kim *et al.*, 2014; Yoshida *et al.*, 2017).

Accurate prediction of the efficacy of drug pairs is thus vitally important, because failures will result in a multidrug resistant phenotype, rather obstructing therapy.

Mathematical models can contribute to a better understanding of the evolutionary dynamics (Wahl & Krakauer, 2000; Traulsen & Hauert, 2009; Song *et al.*, 2015) and may ultimately enable the quantitative prediction of treatment outcomes (Friedman, Higgins, & Gore, 2017). In Chapter 3, we applied evolutionary models to investigate unexpected system behaviors. We had empirically observed low isolate diversity after fast switching treatments, which was opposite to our expectations. To gain a better understanding of the evolutionary dynamics during sequential treatments, we developed a deterministic mathematical model with evolving populations composed of mixture of different genotypes (drug-susceptible or various resistant genotypes). The genotypes were defined by growth rates, resistance, collateral sensitivity and response to antibiotic hysteresis. Intriguingly, this simple model connected the observed diversity to the activity of antibiotic hysteresis. Furthermore, the deterministic dynamics were sufficient to predict the evolved resistance levels (Chapter 3). Although, deterministic models are helpful, they do not reflect the stochastic nature of evolution, which is amplified by bottlenecks in serial transfer experiments (Wahl, Gerrish, & Saika-Voivod, 2002). Individual-based models with finite population size are a more appropriate representation, and can help to quantitatively assess the probability of populations ending in distinct pre-defined states, *e.g.* resistance against single or multiple antibiotics, and even antibiotic tolerance. Stochastic models may thus help to identify treatments with a high probability of favorable evolutionary outcomes.

A cellular perspective

The insights gained in this dissertation highlight the importance of physiological balance and thus homeostasis for bacterial evolution. Antibiotic priming was able to induce hypersensitivity to other antibiotics. Targeting the expression of intrinsic resistance determinants is key for the treatment of already resistant pathogens, which is becoming increasingly important due to the alarming spread of resistance. A promising research frontier may thus be the investigation of the regulation of intrinsic resistance determinants in pathogens. This research will help to understand the molecular mechanisms for antibiotic hysteresis and collateral sensitivity. Genomic data and literature suggest that in *P. aeruginosa* collateral sensitivity is caused by deviation from the natural balance in efflux pump expression. The expression of efflux systems is tightly interconnected; overproduction of certain pumps causes down-regulation of others (Li, Elkins, & Zgurskaya, 2016). Furthermore, their regulation is dependent on AmpC (Masuda *et al.*, 2001), another important resistant determinant. Existing literature is a piecework of case studies, and a systems biology approach may help to understand these complex regulatory modules in their entirety. Thus, we may also begin to better understand the natural function of efflux pumps in the ecology of bacteria.

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List of abbreviations

CAR	carbenicillin
CIP	ciprofloxacin
CEF	cefsulodin
CF	cystic fibrosis
cfu	colony forming units
CI95	confidence interval 95%
CTAB	cetyl trimethyl ammonium bromide
DMSO	dimethyl sulfoxid
DNA	deoxyribonucleic acid
DOR	doripenem
GEN	gentamicin
IC75	inhibitory concentration 75
MIC	minimal inhibitory concentration
MDR	multidrug resistance
ppGpp	alarmone guanosin-3'5'-bispyrophosphat
PIT	piperacillin and tazobactam
RNA	ribonucleic acid
SEM	standard error of the mean
SNP	single nucleotide polymorphism
STR	streptomycin
TOL	antibiotic tolerance

Special devices, materials and chemicals

Plate readers	BioTek Instruments, USA; Ref. EON
Microplate Shakers	Heidolph Instruments, Germany; Ref. Titramax 100, 1mm orbital
Flow Cytometer	Guava EasyCyte HT Blue-Green, Merck KGaA, Darmstadt, Germany 40mW 532nm green laser, 75 mW 488 nm blue laser
Climate chamber	for incubation agar plates 35°C, 60% relative humidity

96-well plates (Greiner Bio-One, Germany; Ref. 655161)

Optically clear, sterile sealing foil (Sarstedt, Germany; Ref. 95.1994)

Freezing foil AlumasealCS, DMSO-resistant (Sigma-Aldrich, USA; Ref. Z722642-50EA)

Table 1. Chemicals relevant to doctoral thesis.

Name	Chemical	Stock mg/ml	Solvent	Storage	Order information	Typical working concentration
CAR	Carbenicillin disodium salt	50	50% Ethanol	prepare fresh	Roth Ref. 6344.2	IC ₇₅ = 50 µg/ml
CIP	Ciprofloxacin	25	0.1M HCl	-20°C	Sigma-Aldrich Ref. 17850-5G-F	IC ₇₅ = 40 ng/ml
CEF	Cefsulodin sodium salt	20	water	-20°C, light sensitive	Roth Ref. 4014.2- 250MG	IC ₇₅ = 0.4 µg/ml
DOR	Doripenem monohydrate	25	water	-20°C	Sigma-Aldrich Ref. 32138-25MG	IC ₇₅ = 30 ng/ml
GEN	Gentamicin solution	50	comes in solution	4°C	Roth; Ref. HN09.1	IC ₇₅ = 480 ng/ml
PIP	Piperacillin sodium salt	50	water	prepare fresh	Sigma-Aldrich Ref. P8396-1G	IC ₇₅ = 1.2 µg/ml
TAZ	Tazobactam sodium salt	10	water	4°C	Sigma-Aldrich Ref. T2820-10MG	4 µg/ml
STR	Streptomycin sulfate salt	25	water	-20°C	Sigma-Aldrich Ref. S6501-5G	IC ₇₅ = 11 µg/ml
PI	Propidium iodide	1.25	water	-20°C	Sigma-Aldrich Ref. P4170-25MG	12.5 µg/ml

Minimal medium M9, defined growth medium for *P. aeruginosa* with glucose (2g/l), citrate (0.5g/l) and casamino acids (1g/l), prepared from two separately autoclaved stocks (Parts A, B), sterile-filtered glucose and casamino acids, and autoclaved deionized water.

- Part A (50x) per liter 350g K₂HPO₄, 100g KH₂PO₄

- Part B (50x) per liter 29.4g Na₃(citrate) x 2 H₂O, 50g (NH₄)₂SO₄, 5g/l MgSO₄ x 7 H₂O

- Glucose (100x), 50ml water, 10g D(+)-glucose monohydrate (Merck; Ref. 1.08342.1000)

- Casamino acids (100x), 50ml water, 5g casamino hydrolysate (Roth; Ref. AE41.1)

Lysogeny broth (LB), Lennox 5 g/l NaCl (Roth; Ref. X964.2)

Lysogeny broth agar (LA plates), Lennox 5 g/l NaCl, 1.5% Agar-Agar (Roth; Ref. X965.3)

Saline, 0.85% NaCl

PBS, phosphate buffered saline, per liter 8.77g NaCl, 2.24g KCl, 0.69g NaH₂PO₄ x H₂O, 0.89g Na₂HPO₄ x 2H₂O, set to pH 7.0

Cfu-counting: LA plates, samples spread by glass beads (~20 per plate)

Freezing bacteria: -80°C in 10% DMSO (v/v)

Polymerase Chain Reaction (PCR): Phusion polymerase (Thermo Fisher; F530S)

DNA isolation: CTAB protocol, as modified by Carola Petersen

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Curriculum vitae

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