

# An evolutionary perspective on antibiotic treatment and the spread of resistance

## **Dissertation**

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## **Declaration**

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I, Aditi Batra, 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 "Contribution of authors";

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

Apart from the included published papers no other part of the thesis has been published nor submitted for publishing;

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

No academic degree of mine was ever withdrawn.

Signature: \_\_\_\_\_



“The important thing is not to stop questioning. Curiosity has its own reason for existence. One cannot help but be in awe when one contemplates the mysteries of eternity, of life, of the marvellous structure of reality. It is enough if one tries merely to comprehend a little of this mystery each day.”

Albert Einstein





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Aditi

## Summary

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Antibiotic resistance at its core is a problem of evolutionary biology. Bacteria evolve resistance to antibiotics either through the accumulation of resistant variants or of horizontally transferred genes. The selection of these resistance determinants over 80 years of the mass use and misuse of antibiotics has resulted in their widespread dispersal, rendering many antibiotics ineffective. While a multifaceted approach is required to solve this crisis, for a sustainable solution all facets must take evolution into account. In this thesis, I approach the fields of antibiotic treatment and the spread of resistance from an evolutionary perspective.

The first part of this thesis focuses on the evolution-informed treatment of *Pseudomonas aeruginosa* infection. *P. aeruginosa* is an opportunistic pathogen of humans, it is particularly dangerous to immunocompromised patients and those with cystic fibrosis, in whom it infects the airways. Antibiotic resistance evolution in this pathogen is well documented and poses a substantial clinical challenge. Evolution-informed antibiotic treatments aim to use evolutionary principles to slow or prevent the acquisition of antibiotic resistance by imposing adaptive constraints on bacterial evolution. These are achieved either by interactions between included antibiotics or through treatment design. Infections with *P. aeruginosa* present an excellent system for evaluating and using such treatments.

In the first chapter, I assessed the efficacy of sequential treatment with  $\beta$ -lactam antibiotics alone, on constraining adaptation in the *P. aeruginosa* strain PA14. Historically, sequential treatment with antibiotics from the same class has been avoided due to the expectation of cross-resistance, however, we found that not only were the triple  $\beta$ -lactam treatments able to eradicate a significant percentage of bacteria but that they did so due to a lack of cross-resistance between the antibiotics and a low spontaneous rate of resistance on one of the antibiotics. Our results emphasize the importance of collateral responses between antibiotics as an adaptive constraint in sequential therapy and introduce the spontaneous rate of resistance as an important predictor of sequential therapy success. Furthermore, they show that the available antibiotics offer unexplored, highly potent treatment options that can be harnessed to counter the spread of drug resistance.

Infections with *P. aeruginosa* can be caused by a variety of diverse strains. This diversity has not been well studied when evaluating evolution-informed treatments. In chapter two, we evaluated whether our prior finding of collateral sensitivity to the aminoglycoside antibiotics due to resistance evolution to ciprofloxacin was robustly reproduced in a strain panel representative of the genetic diversity of the species. We found that the observed collateral sensitivity was conserved across the various strains representing the entirety of genetic diversity in the species and across both spontaneous mutants and long-term evolved populations. This tradeoff, therefore, holds great potential in the treatment of *P. aeruginosa* infection.

Moreover, a single infection with *P. aeruginosa* frequently involves multiple strains and the interaction between strains have consequences for single antibiotic treatment. In chapter three, we evaluated the effect of these interactions on evolution-informed treatments and found that these treatments were able to constrain adaptation in a mixed population of *P.*

*aeruginosa*. The success of these treatments was potentiated by the inclusion of the antibiotic piperacillin/tazobactam. This study is an important step in translating these treatments to the clinic.

In the second part of my thesis, I moved on to assessing the drivers of horizontal gene transfer in Enterobacteriaceae genera - *Escherichia*, *Salmonella*, and *Klebsiella*. We found that the distribution of resistance genes between the plasmid and the chromosome varied contingent on their role in the cell. Genes that had other roles in addition to antibiotic resistance tended to be enriched on the chromosome whilst more specialized resistance genes were more likely to be located on plasmids. We speculate that the distribution and therefore their propensity for horizontal transfer is affected by their degree of connectivity in the cellular network. Highly interconnected genes tend to enrich on the more stable replicon, the chromosome and therefore transfer less.

Taken together, the insights from this thesis not only advance the field of evolution-informed therapy but also contribute to furthering basic research on evolutionary topics.

## Zusammenfassung

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Evolutionsbiologische Prozesse stehen im Zentrum der Antibiotikaresistenzproblematik. Bakterien evolvieren Resistenzen entweder durch Resistenzmutationen oder durch horizontal übertragene Gene. Die Selektion dieser Determinanten durch 80 Jahre des massenhaften Einsatzes und Missbrauchs von Antibiotika hat zu ihrer weiten Verbreitung geführt und viele Antibiotika unwirksam gemacht. Um diese Krise zu lösen ist ein interdisziplinärer Ansatz erforderlich, wobei für eine nachhaltige Lösung evolutionäre Aspekte berücksichtigt werden müssen. In dieser Dissertation untersuche ich Antibiotikabehandlungen und die Ausbreitung von Resistenzen aus einer evolutionären Perspektive.

Der erste Teil dieser Arbeit befasst sich mit der evolutionsbasierten Behandlung von *Pseudomonas aeruginosa*, einem opportunistischen Krankheitserreger des Menschen, der besonders für immungeschwächte Patienten und Menschen mit Mukoviszidose gefährlich ist, da er die Atemwege infiziert. Resistenzentwicklung ist bei diesem Erreger gut dokumentiert und stellt eine klinische Herausforderung dar. Evolutionsbasierte Behandlungen beruhen auf der Nutzung evolutionärer Prinzipien, um den Erwerb von Antibiotikaresistenzen zu verlangsamen oder zu verhindern, indem adaptive Beschränkungen auferlegt werden. Dies wird entweder durch Interaktionen zwischen Antibiotika oder durch die Gestaltung der Behandlung erreicht. Infektionen mit *P. aeruginosa* stellen ein hervorragendes System zur Bewertung und Anwendung solcher Behandlungen dar.

Im ersten Kapitel habe ich die Beschränkung der Anpassung von des *P. aeruginosa*-Stammes PA14 unter einer sequentiellen Behandlung mit  $\beta$ -Laktam-Antibiotika untersucht. In der Vergangenheit wurden wegen erwarteter Kreuzresistenz Behandlungen mit Antibiotika derselben Klasse vermieden. Wir fanden jedoch heraus, dass die dreifache  $\beta$ -Lactam-Behandlung nicht nur einen signifikanten Anteil der Bakterien eliminieren konnte, sondern dass dies auch auf das Fehlen einer Kreuzresistenz zwischen den Antibiotika, und eine geringe spontane Resistenzrate gegen einen der Stoffe zurückzuführen war. Unsere Ergebnisse unterstreichen die Bedeutung von kollateralen Effekten zwischen Antibiotika als adaptive Einschränkung bei der sequentiellen Therapie, und beschreiben die spontane Resistenzrate als wichtigen Prädiktor für den Erfolg sequentieller Therapie.

Infektionen mit *P. aeruginosa* können durch eine Vielzahl unterschiedlicher Stämme verursacht werden. Diese Vielfalt ist bei der Bewertung evolutionsbasierter Behandlungen bisher nicht gut untersucht worden. Im zweiten Kapitel untersuchten wir, ob die zuvor festgestellte kollaterale Empfindlichkeit gegenüber Aminoglykosid-Antibiotika bei einer Resistenz gegen Ciprofloxacin in einer für die genetische Vielfalt der Spezies repräsentativen Stammsammlung robust reproduziert werden kann. Wir stellten fest, dass der beobachtete Effekt über die verschiedenen Stämme und Mutationsverläufe hinweg konserviert war. Dieser Trade-off birgt großes Potenzial für die Behandlung von *P. aeruginosa*-Infektionen.

Darüber hinaus sind an einer Infektion mit *P. aeruginosa* häufig mehrere Stämme beteiligt, deren Interaktionen Konsequenzen für die Therapie haben. In Kapitel drei haben wir die Auswirkungen dieser Interaktionen auf evolutionsbasierte Behandlungen untersucht und festgestellt, dass diese Behandlungen in der Lage sind, die Anpassung auch in einer gemischten

Population von *P. aeruginosa* einzuschränken. Dies ist ein wichtiger Schritt, um diese Therapien in die Klinik zu übertragen.

Im zweiten Teil meiner Arbeit untersuchte ich die Triebkräfte des horizontalen Gentransfers in drei Gattungen der Enterobacteriaceae - *Escherichia*, *Salmonella* und *Klebsiella*. Wir fanden heraus, dass die Verteilung von Resistenzgenen zwischen Plasmiden und Chromosom je nach ihrer zellulären Aufgabe variiert. Gene, die neben der Antibiotikaresistenz noch andere Aufgaben haben, waren eher chromosomal lokalisiert, während spezialisierte Resistenzgene eher auf Plasmiden zu finden waren. Wir vermuten, dass die Verteilung und damit die Neigung zur horizontalen Übertragung vom Grad der Vernetzung im zellulären Netzwerk abhängt. Stark vernetzte Gene werden eher nicht übertragen und reichern sich auf Chromosomen an.

Insgesamt bringen die Erkenntnisse aus dieser Arbeit nicht nur das Feld der evolutionsbasierten Therapie voran, sondern tragen auch zur Förderung der Grundlagenforschung zu evolutionären Themen bei.



## Contribution of authors

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### Chapter 2

#### **High potency of sequential therapy with only $\beta$ -lactam antibiotics.**

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AB, RR, and HS conceived the study. RR performed and analyzed the evolution experiment. AB performed the resistance measurements which were analyzed by RR. AB performed and analyzed the hysteresis experiments. AB, HS, and SF designed the sequencing experiments which were then carried out by SF and analyzed by AB. AB and ER designed and carried out the fluctuation assay which was analyzed by ER. AB designed, carried out, and analyzed the cross-resistance assays with technical help. HS and SF supervised the work and provided funding. AB, RR, and HS wrote the manuscript. All authors read and approved the final manuscript.

### Chapter 3

#### **Robust collateral sensitivity to aminoglycoside antibiotics due to ciprofloxacin resistance evolution in *Pseudomonas aeruginosa*.**

*Adivi Batra*, *Daniel Schuetz*, *Kim Flinders*, *Nadja Steinbach*, and *Hinrich Schulenburg*<sup>#</sup>

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AB and HS conceived the study. AB designed all the experiments and supervised KF and NS who performed them. DS and AB performed the statistical analysis. HS provided supervision and funding. AB and HS wrote the manuscript. All authors read and approved the final manuscript.

### Chapter 4

#### **Evolution-informed therapy constrains adaptation in a genetically diverse population of *Pseudomonas aeruginosa*.**

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AB and HS conceived the study. GS performed and analyzed the experiments with conditioned medium. AB, LT, and HS designed the pre-experiments and the main evolution experiment. TL and AB performed pre-experiments for the main evolution experiment. AB, TL, and KH performed the main evolution experiment which AB analyzed. KH and AB performed the resistance measurements and AB analyzed them.

HS and DU provided supervision and funding. AB and HS wrote the manuscript. All authors read and approved the final manuscript.

## Chapter 5

### **Gene sharing among plasmids and chromosomes reveals barriers for antibiotic resistance gene transfer**

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YW and TD conceived the study. YW performed data analysis and visualization. AB analyzed distribution patterns in ABR gene families. All authors interpreted the results and wrote the manuscript.

I confirm the above stated contributions.

Signature of the supervisor

(Prof. Dr. Hinrich Schulenburg)

Signature of the student

(Aditi Batra)

# INTRODUCTION

## **The rise and fall of antibiotics**

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The term ‘antibiotic’ was coined in 1941 by Nobel laureate Selman Waksman and colleagues<sup>1</sup>. It described chemical compounds that could kill, or inhibit the growth of microbes by targeting essential cellular functions<sup>2,3</sup>. At that point in history, humanity had already witnessed the curative potential of two synthetic antibiotics, Salvarsan and Prontosil, and was on the verge of testing its first microbially-derived antibiotic, Penicillin, on humans<sup>4,5</sup>. What followed the successful testing and mass production of Penicillin was the golden age of antibiotic discovery, wherein most of the antibiotics used today were found<sup>6</sup>. With the advent of these wonder drugs in medicine, the threat of infectious diseases decreased significantly<sup>4</sup>.

The majority of the antibiotics in circulation today are microbially derived<sup>7</sup>. Microbial production of antibiotics long predates their adaption into human medicine. Microbes have been using antibiotics for billions of years for a variety of purposes<sup>8</sup>. These range from their use as weapons for interference competition to signal molecules for quorum sensing, virulence, and host-parasite interactions<sup>2,9</sup>. Further, concomitant to the production of antibiotics, microbes also possess mechanisms that protect them from antibiotic action and confer resistance. These resistance mechanisms are very often clustered together with antibiotic biosynthetic pathways and have likely co-evolved<sup>3</sup>.

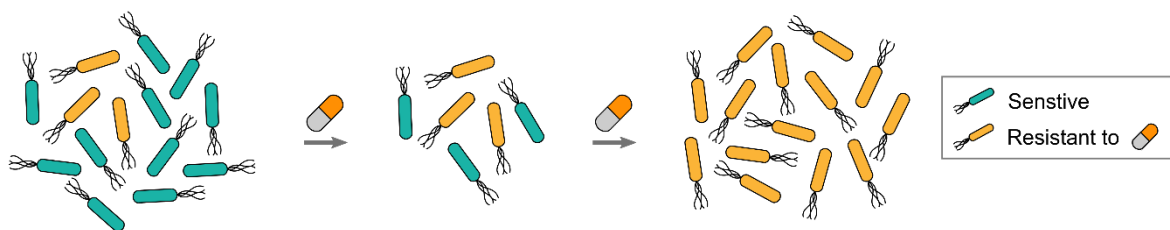
The last 80 years have seen extensive antibiotic use in medicine and agriculture<sup>6,8</sup>. While antibiotic use to treat and prevent infections has been a breakthrough<sup>10</sup>, a lot of antibiotics have been misused. This includes but is not limited to unnecessary prescription of antibiotics for self-limiting infections<sup>11</sup>, use of antibiotics as growth promoters in agriculture<sup>12</sup>, and self-dosing in countries where the antibiotic prescription is not regulated<sup>13</sup>. Further, used antibiotics often end up in the environment where they exert selection on environmental microbes<sup>14</sup>. As a consequence of all this, there has been a selection for resistance determinants against antibiotics<sup>14-16</sup>, rendering them ineffective. What really aggravates the issue, however, is the fact that the rate of introduction of new antibiotics has declined due to scientific and economic barriers<sup>17</sup>. This has left us with a dwindling supply of arsenal against infectious diseases<sup>18,19</sup>. An antimicrobial resistance (AMR) crisis is upon us. A recent study estimated approximately 4.95 million deaths worldwide associated with bacterial AMR in 2019<sup>20</sup>. This number is expected to rise to 10 million a year by 2050 unless immediate action is taken<sup>21</sup>.

### **Antibiotic resistance: Through an evolutionary lens**

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Theodosius Dobzhansky famously said, “Nothing in biology makes sense except in the light of evolution”<sup>22</sup>. Antibiotic resistance is no different; at its core, it is an evolutionary phenomenon<sup>23</sup>. Antibiotics decrease the fitness of bacteria and thereby exert selection for survival. Bacteria can overcome this antibiotic selective pressure through the evolutionary process of natural selection – either through the accumulation of resistant variants<sup>24</sup> or of horizontally transferred genes<sup>25</sup>.

Bacteria can acquire a variety of genetic abnormalities such as base substitution, insertion/deletion (INDEL) of bases, and copy number changes through erroneous DNA replication or exposure to mutagens<sup>26</sup>. These changes can be deleterious or confer benefits such as antibiotic resistance<sup>27</sup>. When a bacterial population is exposed to antibiotic selection, bacteria possessing these changes and thereby resistance, are able to survive while the others die out. Despite the continual application of antibiotics these resistant bacteria thrive as they face no selection and ultimately reconstitute the bacterial population (Fig. 1, 24,28). It is important to note that these genetic abnormalities need not be pre-existing but can arise during treatment<sup>29</sup>. Antibiotic resistance evolution through the accumulation of resistant variants is best exemplified by the evolution of rifampicin resistance in *Mycobacterium tuberculosis* (TB)<sup>30</sup>. Rifampicin is an antibiotic that was demonstrated to be effective against TB in the 1960s<sup>31</sup>. It acts by targeting the  $\beta$ -subunit of the RNA polymerase, where it binds and inhibits the elongation of RNA transcription<sup>32</sup>. Studies from clinical isolates of *M. tuberculosis*<sup>33</sup> and other organisms<sup>34</sup> have shown that rifampicin resistance is nearly always due to point mutations affecting a relatively small part of the  $\beta$ -subunit of RNA polymerase close to the catalytic centre of the enzyme.



**Figure 1: Selection for antibiotic resistance**

Exposure of a bacterial population to an antibiotic kills the sensitive bacteria while the pre-existing resistant bacteria survive. Upon continual exposure to the same antibiotic, the resistant bacteria keep dividing as their fitness is unaffected and ultimately reconstitute the population. The resistance could arise due to genetic variants or horizontally transferred genes. Importantly, resistance need not be present before exposure to antibiotic and can arise during antibiotic exposure.

Transfer of genetic material need not always be vertical and can also happen between more distantly related lineages and between species. Such a kind of transfer is termed horizontal gene transfer (HGT)<sup>35</sup>. Horizontal transfer of genetic material requires that it be transferred into a cell and then be stably maintained<sup>8</sup>. Transfer can be of two types. The first type requires cell-cell contact and consists of conjugation mediated through conjugative plasmids and transposons<sup>36</sup>. The second type is independent of cell-cell contact and consists of DNA being taken up from outside the cell through transformation or being shuttled between cells through bacteriophages<sup>37–39</sup>. Many factors influence the mode of HGT in play, including but not limited to the environment of the cell, the cell density, compatibility of phage and plasmid types, etc. Once the genetic material is acquired it can be stably maintained either through the origin of replication as in the case of plasmids or through integration into the recipient genome for other mobile elements<sup>8</sup>. Although the rates of HGT can differ across species, in some taxa, several HGT events can occur during the time that takes for the genome to accumulate, on average, one nucleotide substitution per gene<sup>40</sup>. Antibiotic resistance genes

transmitted through HGT are enriched by antibiotic selection, similar to resistance-conferring variants found on the chromosome<sup>8</sup>. A classic example of a horizontally transferred antibiotic resistance gene is the penicillinase of *Staphylococcus aureus*<sup>41</sup>. Penicillinase is a beta-lactamase enzyme that inactivates the antibiotic penicillin<sup>42</sup>. Prior to the introduction of penicillin, *S. aureus* bacteraemia was a major cause of mortality. However, with the introduction of the antibiotic in the early 1940s, patient survival drastically improved. This success was however short-lived; penicillinase containing *S. aureus* was discovered in 1942 and within the next 20 years spread throughout the community<sup>41</sup>.

What kind of genetic variation can evolution act on to select for antibiotic resistance? Antibiotic resistance can involve a variety of genes clustered into different mechanisms of resistance<sup>43</sup>. Bacterial cells can evolve resistance to an antibiotic by reducing its influx into the cell or increasing its efflux so as to reduce the intracellular concentration of the antibiotic, by modifying the antibiotic's cellular target through genetic mutation or post-translational modification to prevent antibiotic binding, through inactivation of the antibiotic or by replacement of the original susceptible target by a resistant one<sup>44</sup>. While certain types of mechanisms are enriched on chromosomes and others on mobile genetic elements, some are found on both<sup>45</sup>. I discuss the possible determinants for such a distribution of AMR mechanisms in **Chapter 5** of this thesis.

### **Social interactions conferring antibiotic resistance**

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Bacteria can survive antibiotic exposure not only through the mechanisms mentioned above but also through social interactions<sup>46</sup>. A prime example of this is the production of antibiotic-inactivating enzymes. These enzymes decrease the effective amount of antibiotic around the bacteria by degrading it; thereby reducing its inhibitory effect<sup>47</sup>. This not only benefits the bacteria producing these enzymes but also those which do not produce them but are in the vicinity of the producers. Therefore, they can survive antibiotic exposure without being genetically resistant<sup>48</sup>. For example, the enzymatic inactivation of ampicillin via  $\beta$ -lactamase producing *Escherichia coli* can protect *Salmonella enterica* serovar Typhimurium from lethal concentrations of the antibiotic<sup>49</sup>.

Another example of increased resistance without genetic changes are biofilms. These are microbial aggregates in which the cells are held together by a self-secreted polymer matrix of exopolysaccharides, DNA and proteins<sup>48,50</sup>. These aggregates can be of a single species or a multi-species community<sup>50,51</sup>. Biofilms can confer antibiotic resistance in a multitude of ways. The polymer matrix acts as a barrier to the diffusion of the antibiotic, thereby protecting the cells contained within a biofilm<sup>52</sup>. Some bacteria within a biofilm can also produce antibiotic-inactivating enzymes, thus protecting themselves and the other members<sup>53</sup>. In addition, the centres of biofilms are deprived of nutrients and oxygen. This induces a metabolically tolerant state in the cells at the centre further protecting them from antibiotic action, which requires the cells to be actively dividing<sup>54</sup>. Indeed, the presence of biofilms makes infections difficult to treat<sup>55</sup>.

Sometimes, bacteria may carry resistance genes but require interactions with other bacteria to express them<sup>46,48</sup>. This is the case with the indole-dependent multi-drug efflux pump

in *Pseudomonas putida*. Though it carries the pump, *P. putida* is incapable of producing indole. Secretion of indole by *E. coli* has been shown to activate the expression of the pump resulting in elevated antibiotic resistance in *P. putida*<sup>56</sup>.

Although many social interactions can confer antibiotic resistance some are detrimental to it. Cross-feeding creates metabolic dependencies between the participating bacteria<sup>57</sup>. In such a network, the antibiotic resistance of the collective is reduced to that of the least resistant member – the weakest link. Antibiotic concentrations enough to kill the weakest link can eradicate the whole network<sup>58</sup>. Dependence on susceptible bacteria, therefore, compromises the survival of resistant bacteria. Competitive interactions between bacteria can elevate the cost of resistance genes<sup>59,60</sup>. This has been shown to constrain the growth of resistant bacteria in communities<sup>59</sup> but also restrict the *de novo* evolution of resistance in focal strains<sup>61</sup>.

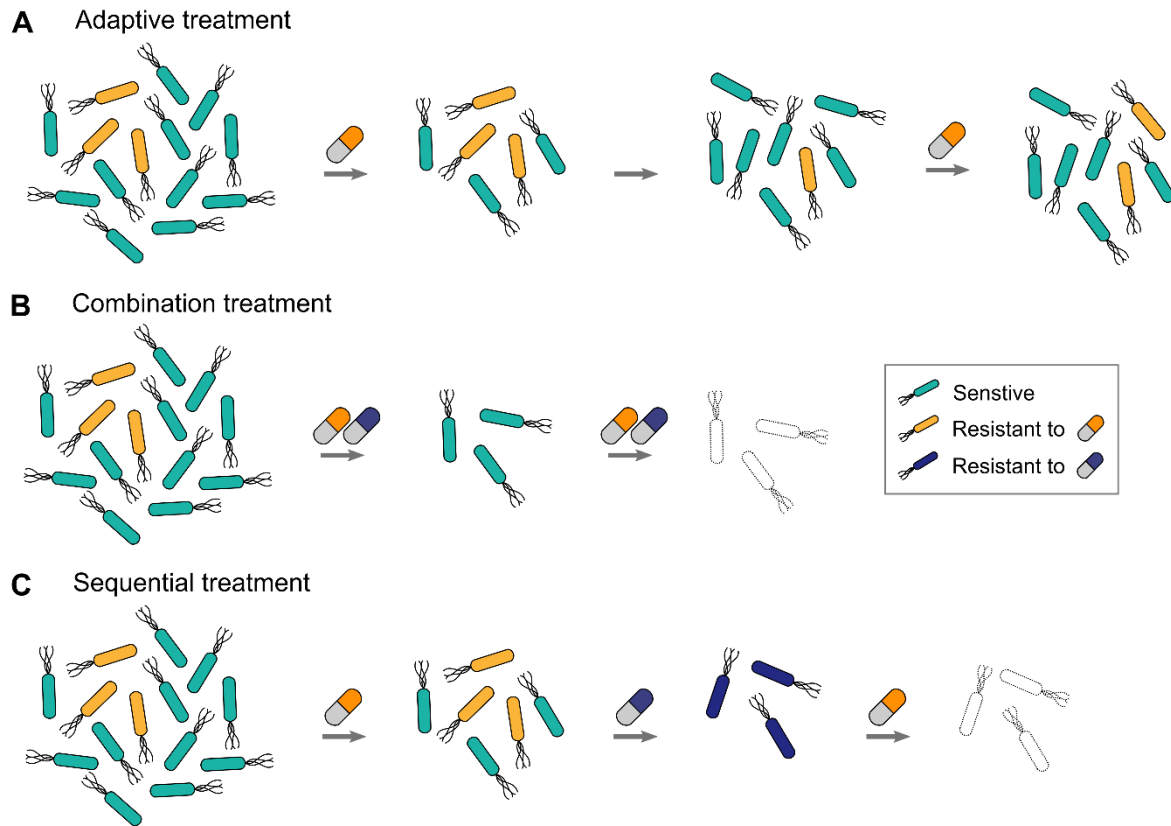
### **Taking an evolutionary approach to antibiotic therapy**

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Tackling the antimicrobial resistance crisis requires approaching the problem from many angles<sup>21</sup>. As a society, we must try and reduce our dependence on antibiotics so that the current stocks can last longer. This includes creating global awareness of the crisis, improving sanitation to prevent the spread of infection, promoting rapid diagnostics to reduce the unnecessary use of drugs in medicine, promoting the development of vaccines against infections, and reducing the unnecessary use of antibiotics in agriculture and their dissemination into the environment. We must also try and increase the supply of new antibiotics that can target drug-resistant bacteria. This would be aided by diverting government funds into early-stage and non-commercial research for new drugs and having better incentives for drug development<sup>14,21</sup>. These different approaches while essential, however, do not target the process of resistance evolution itself. Even if new drugs are introduced, resistance evolution to them will follow, as has been the case till now<sup>62</sup>. These approaches must be complemented with treatments that select against resistance; only then will we be able to use our antibiotic arsenal sustainably.

Evolutionary biologists have utilized evolutionary concepts to design antibiotic treatments that either reduce the likelihood of *de novo* resistance evolution and/or impose selection against evolved resistance<sup>23,24,63–65</sup>. An example of these so-called evolution-informed treatments is adaptive treatment. Standard clinical practice demands that infections be treated as aggressively as possible, with the aim of eradicating bacteria before the *de novo* evolution of resistance<sup>66</sup>. If, however, the initial bacterial population contains resistant bacteria, aggressive treatment eliminates the sensitive population resulting in the competitive release of the resistant bacteria<sup>67,68</sup>. Adaptive treatment aims to avoid this by using a containment strategy. This strategy tries to contain the spread of resistant bacteria by competitively suppressing them with sensitive bacteria (Fig. 2A). This increases the time taken for the resistant population to expand<sup>65</sup>. While adaptive treatment has only recently been

experimentally tested in bacterial infections, the treatment strategy has shown promising results in the treatment of cancer and malarial parasites<sup>69,70</sup>.



**Figure 2: Evolution-informed antibiotic treatments**

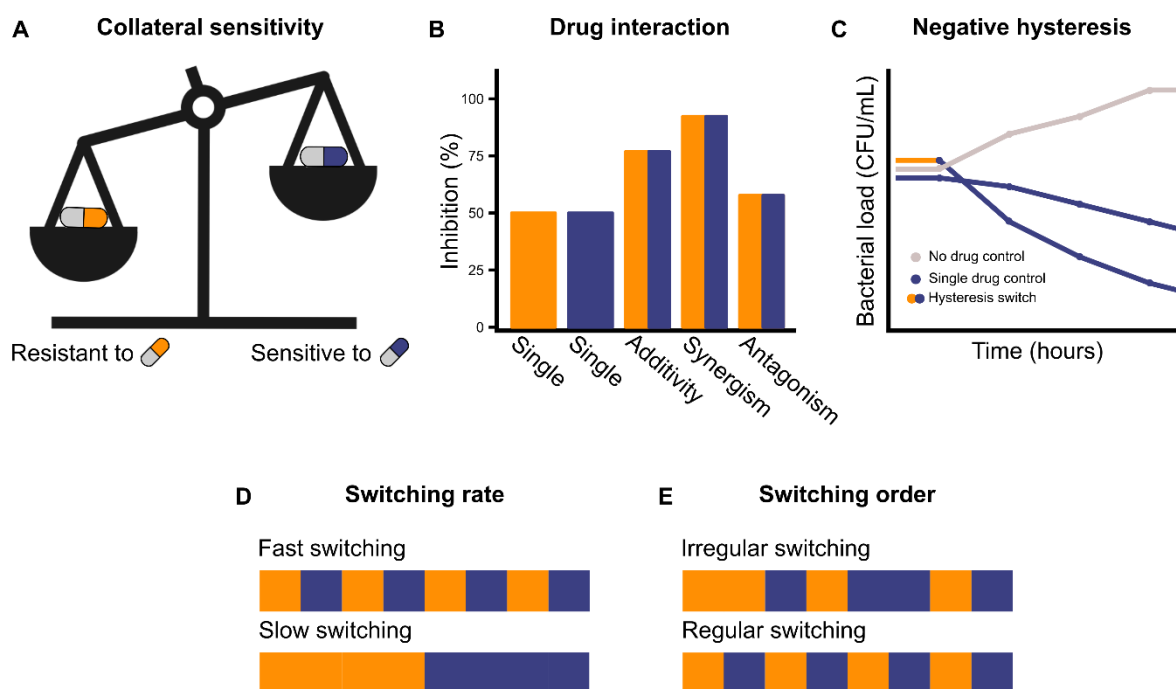
Evolution-informed treatments are designed using evolutionary concepts to select against resistance evolution. **(A)** Adaptive treatment aims to contain resistant bacteria by competitively suppressing them using sensitive bacteria. To do so it cycles between drug and no-drug periods. The drug period selects for resistant bacteria, however, in the no-drug period the resistance is costly and results in the resistant bacteria being outcompeted by the sensitive bacteria. This leads to the containment of the resistant population. **(B)** Combination treatment reduces the probability of *de novo* resistance evolution. In order to survive the bacteria need to be resistant to both antibiotics simultaneously. This is unlikely unless common resistance determinants are easily accessible and of low cost. As a consequence, the sensitive population eventually dies out. **(C)** Sequential treatment tries to eliminate the resistant bacteria by fluctuating between antibiotics where the probability of cross-resistance is low. Exposure to the first antibiotic selects for resistant bacteria. The resistance mechanism of these bacteria does not confer fitness in the second antibiotic and these bacteria die out.

Another evolution-informed treatment strategy is to combine different drugs. The rationale behind it is that the probability of evolving resistance to both drugs is very low in the absence of common resistance determinants (Fig. 2B)<sup>64</sup>. Furthermore, by combining drugs the treatment can target more bacteria if the cause of the infection is not yet known<sup>71,72</sup>. Combination therapy can be potentiated by phenomena that act as adaptive constraints. One such constraint is collateral sensitivity between the combined antibiotics. Collateral sensitivity is the evolved hypersensitivity to an antibiotic upon resistance evolution to a different antibiotic<sup>73,74</sup> (Fig. 3A). Combination treatments with antibiotics displaying collateral



sensitivity have been shown to constrain resistance evolution as compared to those which don't<sup>75-77</sup>. Drug synergy is yet another constraint that can impact the success of combination treatments. Some drugs potentiate each other's effect when used together<sup>71</sup>, when such drugs are used together they result in higher bacterial clearance<sup>75</sup> (but also see 78-80, Fig. 3B). Resistance to individual drugs can also predict resistance evolution to the combination<sup>75-77</sup>. Finally, targeting heteroresistant populations in combination therapy has also been shown to constrain the evolution of resistance<sup>81</sup>.

Antibiotics can also be sequentially alternated as an evolution-informed treatment (Fig. 2C). This strategy derives from the concept of fluctuating selection wherein if adaptive mechanisms to two environments are independent, then adaptation to one environment does not confer fitness benefits when the environment is switched<sup>82</sup>. Like combination therapy, sequential therapy also profits from adaptive constraints. Collateral sensitivity has been shown to potentiate sequential therapy by reducing the evolved resistance of the treated bacteria<sup>83-86</sup>, increasing bacterial eradication<sup>83,84,87,88</sup> and/or limiting the rate of adaptation<sup>63,89,90</sup>. Sometimes, short exposure to one antibiotic can increase the killing by another. This phenomenon of negative hysteresis has been shown to constrain the rate of adaptation and multidrug resistance when such switches were employed in fast sequential therapy<sup>91</sup> (Fig. 3C). The efficacy of sequential therapy can be modulated by the type of sequence in addition to the constituent antibiotics. Both the switching rate<sup>89,91,92</sup> as well as the order of the sequence<sup>89,90</sup> have been shown to affect the success of sequential therapy (Fig. 3D and E). In **Chapter 2** of this thesis, I present results that identify an additional determinant of sequential therapy success to those mentioned above.



**Figure 3: Adaptive constraints potentiating evolution-informed treatments.**

Different types of adaptive constraints can be imposed on bacterial evolution either by interactions between included antibiotics or through treatment design. **(A)** Collateral sensitivity is an evolutionary trade-off where the evolution of resistance to one antibiotic results in hypersensitivity to another. This is contrasted by cross-resistance where the evolution of resistance to one antibiotic results in resistance to another. Collateral sensitivity potentiates the efficacy of both – combination and sequential treatments **(B)** Drug interactions as defined by Bliss independence. When antibiotics are administered simultaneously the inhibition varies from single treatment and can either be additive, synergistic, or antagonistic. Synergistic antibiotic combinations can significantly increase the eradication of bacteria. **(C)** Short exposure to one antibiotic can increase the killing by another. Such negative hysteresis interactions between antibiotics can constrain adaptation in sequential treatments. **(D)** The rate of switching in sequential treatments can affect adaptation; fast sequential treatments are more successful at bacterial eradication than slow sequential treatments. **(E)** The order of antibiotics can also constrain adaptation with an irregular order resulting in greater treatment success than a regular order.

Phenomena which can act to constrain adaptation, and thereby potentiate evolution-informed treatments, are not necessarily always robust. For example, the evolution of collateral sensitivity is highly contingent on the mechanism selected for resistance, right down to the exact single nucleotide polymorphism (SNP)<sup>93,94</sup>. Therefore, the repeatability of the phenotype can be compromised due to the stochastic nature of evolution<sup>95</sup>. Further, the phenotype of collateral sensitivity can change over time under treatment as the mutational profile of the population under selection changes<sup>96</sup>. It can also differ across species due to a change in epistatic interactions with the genomic background<sup>97</sup>. Drug interaction and negative hysteresis are no different; they too vary with genetic background and over time under treatment<sup>76,91,98,99</sup>. This complicates treatment as its success relies on these constraints being robust. In the last couple of years, there has been an increased focus on identifying robust adaptive constraints. The field of collateral sensitivity has seen theoretical studies provide indices to quantify the

degree of collateral sensitivity in antibiotic pairs given the stochastic nature of evolution<sup>95,100</sup>. These studies have been complemented with experimental studies reporting robust collateral sensitivity between antibiotic pairs across different AMR mutations and species<sup>84,88,101</sup>. **Chapter 3** of this thesis adds to this body of literature and presents results on a robust collateral sensitivity trade-off useful for the treatment of the opportunistic pathogenic bacteria, *Pseudomonas aeruginosa*. Even though antibiotic synergy is less frequently observed than antibiotic antagonism (where the antibiotics reduce each other's effect) it was found to be more conserved<sup>98</sup>. The same study identified antibiotic pairs that showed conserved synergy across *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Pseudomonas aeruginosa*<sup>98</sup>.

### **Antibiotic resistance in Gram-negative bacteria**

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The many thousands of known bacterial species can be divided into two simple categories based on their cell envelope. Gram-negative bacteria are bound by a cell envelope consisting of an inner membrane, a thin peptidoglycan cell wall, and the outermost membrane containing lipopolysaccharides. These are contrasted with Gram-positive bacteria which do not possess the outermost membrane but have a much thicker peptidoglycan wall<sup>102</sup>. This distinction has serious implications for antibiotic resistance. By reducing permeability, the presence of the outer membrane makes Gram-negative bacteria intrinsically resistant to a number of antibiotics<sup>103,104</sup>. Indeed, four of the six World Health Organization's priority 'ESKAPE' pathogens are Gram-negative bacteria<sup>105</sup>. In addition to intrinsic resistance Gram-negative bacteria can also evolve resistance. This can be due to selection for point mutations or horizontally transferred resistance genes<sup>104</sup>. In particular, the Enterobacteriaceae primarily become resistant by the acquisition of genes from a shared pool<sup>106</sup>.

### ***Pseudomonas aeruginosa* as a model for evolution-informed treatments**

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*Pseudomonas aeruginosa* is a rod-shaped Gram-negative bacterium belonging to the  $\gamma$ -Proteobacteria subclass of the phylum Pseudomonadota (synonym Proteobacteria)<sup>107,108</sup>. Its genome is 5.5-7 megabases large and encodes a large repertoire of transporters, transcriptional regulators, and two-component regulatory systems<sup>109</sup>. This genomic diversity confers to *P. aeruginosa* a wide range of metabolic and physiologic functions, allowing it to colonize several ecological niches including humans<sup>110</sup>. Indeed, *P. aeruginosa* is a notorious opportunistic pathogen causing a number of infections in immunocompromised patients<sup>111</sup>. It is also a leading cause of death in patients with cystic fibrosis (CF)<sup>112</sup>. Though most patients with CF are immunocompetent<sup>113</sup>, the mucus accumulation in the lung due to the defective CFTR gene provides a favourable environment for bacterial growth<sup>114</sup>. Treatment of *P. aeruginosa* infections in the CF lungs often requires prolonged and intensive antibiotic therapy<sup>115,116</sup>. Consequently, antibiotic resistance evolution in *P. aeruginosa* in the CF lung is well documented<sup>15,117</sup>, and makes it an excellent system for evolution-informed antibiotic treatment.

Most infecting bacteria harbour great genotypic and phenotypic diversity. *P. aeruginosa* is no different. Its genome is divided into a core genome, which is relatively conserved across isolates, and a fairly large accessory genome which is variable amongst isolates<sup>118</sup>. Analysis of this variability through a binary marker microarray reveals a population structure of 323 clone types<sup>119,120</sup>. These clone types have been shown to vary in their virulence

phenotypes<sup>121</sup> and their frequency in natural and clinical habitats<sup>120</sup>. Analysis of published genome sequences of *P. aeruginosa* divides it into 3-5 phylogenetic clades of unequal sizes. The genomes further show evolutionary dynamics that are clade-specific<sup>122,123</sup>. The diversity, therefore, has consequences for the evolution and spread of the pathogen. To provide type strains for functional analysis, a recent study sequenced 18 of the most frequent clone types. Strains from this *major Pseudomonas aeruginosa clone type* (mPact) panel together evenly spread out over the phylogeny of all complete published *P. aeruginosa* genomes<sup>122</sup>.

The variability in *P. aeruginosa* species is reflected at the level of the patients. Different patients can be infected with different clone types of *P. aeruginosa*, or a single patient can have multiple clone types infecting it<sup>124–126</sup>. In infections with a single successful epidemic strain, the infecting bacteria can evolve and diversify, forming distinct lineages which can vary from a few to a hundred SNPs and up to 40 INDELS<sup>127–129</sup>. This number can go up to a few hundred SNPs when hypermutator strains are present<sup>129</sup>. Most of this diversity is ignored while evaluating evolution-informed treatments; they are usually tested with single strains of species<sup>75,85,87,130</sup>. In **Chapter 3** of this thesis, I try to bridge this gap by extending the testing of collateral sensitivity constraints to the entire genomic diversity in *P. aeruginosa* using the mPact panel.

The different isolates of *P. aeruginosa* also have different phenotypes. They vary in their antibiotic resistance levels, growth rates, motility, and colony morphology amongst other phenotypes<sup>115,131,132</sup>. All these isolates together form a collective and partake in social interactions. These include the production of  $\beta$ -lactamases<sup>133</sup> and quorum-sensing molecules<sup>134</sup>, the formation of biofilms<sup>135</sup>, and competition between clone types<sup>136</sup>. Social interactions are known to impact conventional antibiotic treatment, with the effect contingent on the type of interaction<sup>59,61,137–139</sup>. This adds another level of complexity to the treatment of bacterial infections. How social interactions affect evolution-informed treatments is mostly ignored. I try to answer this question in **Chapter 4** of this thesis.

## Objectives and overview of this thesis

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In this thesis, I explore antibiotic resistance from an evolutionary viewpoint. The thesis is structured into two parts; the first part addresses questions pertaining to evolution-informed treatments in *Pseudomonas aeruginosa* while the second part looks at the drivers of horizontal gene transfer of antibiotic resistance genes in the Enterobacteriaceae genera *Escherichia*, *Salmonella*, and *Klebsiella*. Finally, in the general discussion at the end, I take insights from my work and contextualize them in current literature. A brief description of each of the chapters in my thesis is given below:

### Part I. Evolution-informed treatments in *Pseudomonas aeruginosa*

#### Chapter 2: High potency of sequential therapy with only $\beta$ -lactam antibiotics

Conventionally, sequential therapy has always been performed with antibiotics targeting different cellular functions. This is because the mechanisms of resistance to these antibiotics are more likely to be independent and resistance to one should not confer fitness benefits in the next. This assumption is partially violated when all the antibiotics target the same function. In

this case, resistance to the first antibiotic has a higher likelihood to provide cross-resistance to the next, resulting in treatment failure. The first chapter of this thesis is a study in which we experimentally test this hypothesis by detailing the evolutionary response of bacteria to sequential therapy with antibiotics which either all target the same cellular function or all different cellular functions. We find that treatments with antibiotics targeting the same cellular function, contrary to expectation, result in a greater clearance of bacteria. We demonstrate that this is due to inclusion of antibiotics in the sequence with lower rates of spontaneous resistance emergence and which have lower likelihoods of cross-resistance. Overall, our study shows that the available antibiotics offer unexplored, highly potent treatment options that can be utilized to counter the spread of drug resistance. It further highlights the importance of the likelihood of cross-resistance in designing sequential treatments and lastly, introduces spontaneous rate of resistance emergence as an adaptive constraint that can enhance the efficacy of sequential treatments.

### **Chapter 3: Robust collateral sensitivity to aminoglycoside antibiotics due to ciprofloxacin resistance evolution in *Pseudomonas aeruginosa***

A previous study from our lab<sup>93</sup> reported that resistance evolution to the fluoroquinolone ciprofloxacin resulted in repeatable collateral sensitivity to the tested aminoglycoside antibiotics in the *Pseudomonas aeruginosa* type strain PA14. In this chapter of the thesis, we wanted to assess if this collateral sensitivity pattern was consistent across genetically different strains of the species and different evolutionary trajectories. This is particularly important as collateral sensitivity interactions are known to vary between genomic backgrounds and between different sets of mutations, which can limit treatment success. We characterized evolved cross-resistance and collateral sensitivity in long-term evolved populations and spontaneous mutants on ciprofloxacin in the mPact panel and the type strains PA14 and PAO1. We found that resistance to ciprofloxacin gave consistent collateral sensitivity to the aminoglycoside antibiotics gentamicin and tobramycin across both, the different backgrounds and the different trajectories. Our results identify a robust adaptive constraint that can be used in combination or sequential treatment of *Pseudomonas aeruginosa* infections.

### **Chapter 4: Evolution-informed therapy constrains adaptation in a genetically diverse population of *Pseudomonas aeruginosa***

Infections with *Pseudomonas aeruginosa* can consist of multiple genetically distinct strains which together form a collective exhibiting higher-order social interactions. These interactions can potentiate or compromise antibiotic efficacy depending on the type of interaction present. Yet, the effect of these interactions on evolution-informed treatments has mostly been ignored. In this chapter of the thesis, we try to bridge this gap by evaluating combination and sequential treatments with two antibiotics, piperacillin/tazobactam, and gentamicin. These two antibiotics were chosen as they exhibit multiple adaptive constraints – drug synergy, reciprocal collateral sensitivity, and negative hysteresis. We find that both treatments increase the time taken for adaptation of bacteria relative to the monotherapies and do not result in increased resistance in most cases. The efficacy of these evolution-informed treatments further depends on the antibiotic piperacillin/tazobactam as adapting to it appears to be difficult. We hypothesize that this is likely due to negative interactions in the population. Our study is the first to assess the

role of social interactions in evolution-informed treatments in a mixed population of a single species.

## **Part II. Drivers of horizontal gene transfer of antibiotic resistance genes**

### **Chapter 5: Gene sharing among plasmids and chromosomes reveals barriers for antibiotic resistance gene transfer**

In the last chapter of my thesis, we characterized the distribution of antibiotic resistance genes (ABR) in the Enterobacteriaceae genera *Escherichia*, *Salmonella*, and *Klebsiella* with the aim of identifying determinants of the horizontal transfer of these genes. We found that the ABR genes were unevenly distributed between the bacterial chromosomes and plasmids with some genes present on both replicons. This distribution further varied with the antibiotic resistance mechanism classes – genes coding for antibiotic efflux were enriched on the chromosome while those coding for antibiotic inactivation/target replacement and target protection were enriched on the plasmid. Genes that were shared between plasmids and chromosomes were shown to be horizontally transferred through the reconstruction of their phylogenies. Since genes like efflux pumps are involved in many cellular processes aside from antibiotic resistance, they are expected to be highly integrated in cellular networks. Loss or gain of such a gene is expected to interfere with the proper functioning of the network. Therefore, such genes are likely to be maintained on more stable replicons, the chromosome, hence, they would be transferred less. This is established for non-ABR genes. Our results show that the barriers that exist for non-ABR genes also extend to ABR genes.

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### HIGH POTENCY OF SEQUENTIAL THERAPY WITH ONLY $\beta$ -LACTAM ANTIBIOTICS

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Evolutionary adaptation is a major source of antibiotic resistance in bacterial pathogens. Evolution-informed therapy aims to constrain resistance by accounting for bacterial evolvability. Sequential treatments with antibiotics that target different bacterial processes were previously shown to limit adaptation through genetic resistance trade-offs and negative hysteresis. Treatment with homogeneous sets of antibiotics is generally viewed to be disadvantageous, as it should rapidly lead to cross-resistance. We here challenged this assumption by determining the evolutionary response of *Pseudomonas aeruginosa* to experimental sequential treatments involving both heterogeneous and homogeneous antibiotic sets. To our surprise, we found that fast switching between only  $\beta$ -lactam antibiotics resulted in increased extinction of bacterial populations. We demonstrate that extinction is favored by low rates of spontaneous resistance emergence and low levels of spontaneous cross-resistance among the antibiotics in sequence. The uncovered principles may help to guide the optimized use of available antibiotics in highly potent, evolution-informed treatment designs.

## Introduction

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The efficacy of antibiotics for the treatment of infections is diminishing rapidly, as bacteria evolve new mechanisms to resist antibiotics (Laxminarayan et al., 2013). Resistance evolution is frequently observed during antibiotic therapy and can happen within days (Bloemberg et al., 2015; Hjort et al., 2020; Tueffers et al., 2019). A failure to account for such rapid bacterial adaptation is likely a common reason for treatment failure (Woods and Read, 2015; Zhou et al., 2020). For this reason, the field of evolutionary medicine specifically accounts for bacterial evolvability and seeks treatment solutions that are hard to overcome by genetic adaptation (Andersson et al., 2020; Merker et al., 2020). While an evolution-proof antibiotic remains to be found, the mechanisms that restrict evolutionary escape are starting to be revealed (Bell and MacLean, 2018). Such evolutionary insight may guide the design of effective and sustainable antibiotic therapy.

An effective way of reducing the amount of evolutionary solutions is to administer several antibiotics either simultaneously (i.e., combination therapy) or sequentially (i.e., sequential therapy). Tailored combination treatments make use of physiological and evolutionary constraints (Baym et al., 2016). The emergence of resistance is delayed by combinations, when evolutionary escape requires multiple mutations and when drug interactions eliminate the intermediate genetic steps of single-drug resistance (Chait et al., 2007), antibiotic tolerance (Levin-Reisman et al., 2017), and heteroresistance (Band et al., 2019). However, when genetic resistance to the combination is easily accessible, for example through gene amplification of efflux pumps, then combination therapy can accelerate the emergence of resistance as a consequence of competitive release (Pena-Miller et al., 2013). These undesired selective effects are potentially avoided by sequential drug application. Evolutionary escape from sequential treatments can be constrained by negative hysteresis responses induced by specific antibiotics (Roemhild et al., 2018) and/or the emergence of genetic collateral sensitivity trade-offs (Barbosa et al., 2019; Yoshida et al., 2017). Negative hysteresis occurs when exposure to an antibiotic induces changes to bacterial physiology that transiently increases the killing efficacy of other antibiotics (Roemhild et al., 2018). Collateral sensitivity is a genetic side-effect of evolved resistance that too increases the efficacy of other antibiotics (Szybalski and Bryson, 1952). Collateral sensitivity is prevalent among pathogens and occurs especially between antibiotics with distinct mechanism of action (i.e., heterogeneous sets of antibiotics), while cross-resistance often emerges towards antibiotics with similar mode of action (i.e., homogeneous sets of antibiotics) (Barbosa et al., 2017; Imamovic and Sommer, 2013; Lazar et al., 2014; Maltas and Wood, 2019). Thus, conventionally, multidrug treatments would avoid antibiotics from similar classes, with the rationale of limiting the overlap in the respective sets of resistance mutations, and thus the ensuing cross-resistance.

The particular efficacy of sequential therapy has been confirmed with the help of evolution experiments under controlled laboratory conditions. Different types of sequential treatments have been tested. Some regimens involved a single switch between antibiotics, while others included multiple switches at short time intervals. One of the main findings was that the efficacy of sequential treatments depended both on the included antibiotics and the

particular treatment sequence (Fuentes-Hernandez et al., 2015; Maltas and Wood, 2019; Roemhild et al., 2015). While fast sequential treatments did not exclude the eventual emergence of multidrug resistance, many significantly delayed bacterial adaptation compared to monotherapy (Kim et al., 2014; Roemhild et al., 2015; Yoshida et al., 2017). A single antibiotic switch can also delay adaptation, dependent on the drug order, and it can additionally reverse previous resistance and re-sensitize bacterial populations to specific antibiotics (Barbosa et al., 2019; Hernando-Amado et al., 2020; Imamovic and Sommer, 2013; Yen and Papin, 2017). Moreover, our group previously demonstrated that fast sequential treatments with a heterogeneous set of three antibiotics – the fluoroquinolone ciprofloxacin (CIP), the  $\beta$ -lactam carbenicillin (CAR), and the aminoglycoside gentamicin (GEN) – delayed the emergence of multidrug resistance in the pathogen *Pseudomonas aeruginosa* (Roemhild et al., 2018). The observed inhibition of evolutionary escape was manifested by the occurrence of population extinction, although antibiotic concentrations were below the minimal inhibitory concentration (MIC). We further found a reduced adaptation rate that was explained by strong selective dynamics at antibiotic switches due to negative hysteresis. Selection favoured distinct genetic changes that predominantly suppressed negative hysteresis and only mildly increased resistance. We assumed that the acting selective dynamics were ultimately a consequence of antibiotic heterogeneity. However, is this so? Do selective dynamics differ for a homogenous set of drugs?

The primary aim of our current study was to assess the efficacy of sequential treatments with either heterogeneous or homogeneous sets of three antibiotics. We performed similar evolution experiments as before, with three new sets of bactericidal antibiotics, two of which included only  $\beta$ -lactams, and one the three previously considered modes of action (Fig. 1, Figure 1-Supplementary Table 1). As before, the heterogeneous drug set CIP, streptomycin (STR), and doripenem (DOR) involved both collateral sensitivity and antibiotic synergy (Barbosa et al., 2018, 2017). The drug sets comprising three  $\beta$ -lactams, however, had all properties that would typically be avoided for the design of multidrug treatments. The three  $\beta$ -lactams carbenicillin (CAR), cefsulodin (CEF), and doripenem (DOR) have the same core structure and individually inhibit the DD-transpeptidase activity in cell-wall synthesis (Walsh, 2003). The collateral effects landscape between CAR-CEF-DOR was dominated by cross-resistance (Barbosa et al., 2017) and the three antibiotics showed neither synergy nor antagonism (Barbosa et al., 2018). Resistance to these antibiotics may potentially be achieved through single mutations. The situation is replicated by the set of ticarcillin (TIC), azlocillin (AZL), and ceftazidime (CEZ). In contrast to expectations, the triple  $\beta$ -lactam sequences showed high treatment potency. Therefore, the secondary aim of our study was to assess which characteristics constrain the ability of the bacteria to adapt to the  $\beta$ -lactam sequential treatments. We focused on one triple  $\beta$ -lactam set (CAR-CEF-DOR) and specifically tested the influence of antibiotic switching rate, switching regularity, negative hysteresis, the potential for spontaneous resistance evolution and resulting cross-resistances on treatment efficacy.

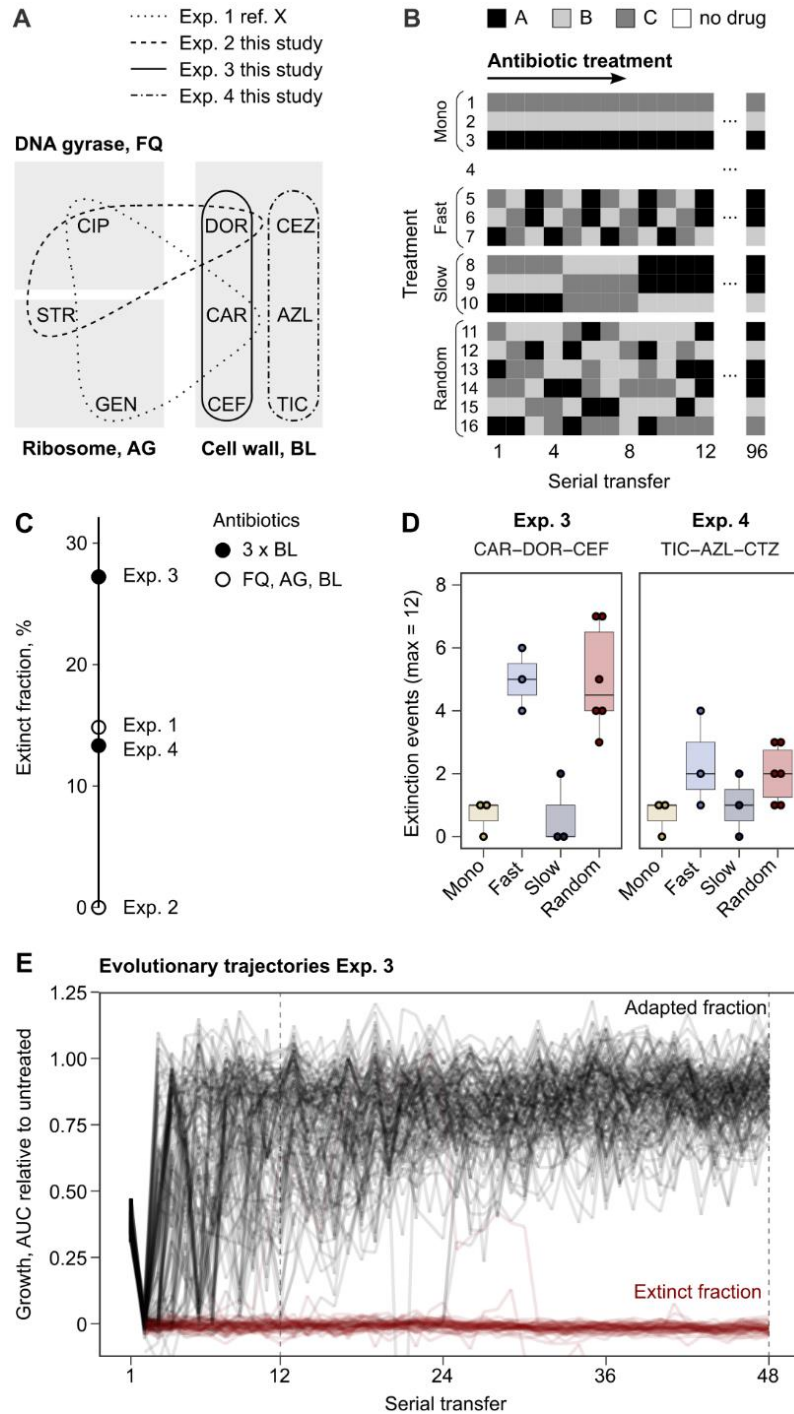
## Results

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### Triple $\beta$ -lactam sequential treatments favor extinction of bacterial populations

We challenged a total of 756 replicate *P. aeruginosa* populations with sequential treatments across three fully independent evolution experiments, each focused on a different set of three antibiotics (Fig. 1, Figure 1-Figure Supplement 1, Figure 1-Supplementary Table 1, Material and methods). The antibiotic concentrations were calibrated to an inhibitory concentration of 75% (IC75), allowing bacteria to adapt to the imposed selection pressure. We used a serial dilution protocol for experimental evolution, with 2% culture transfer after 12 h (one season) across a total of 96 seasons, equivalent to approximately 500 bacterial generations. Following the previous setup (Roemhild et al., 2018), we recorded the evolutionary dynamics in response to 16 different treatments, belonging to four main treatment types: monotherapy, fast-regular, slow-regular, and random sequential therapy (Fig. 1).

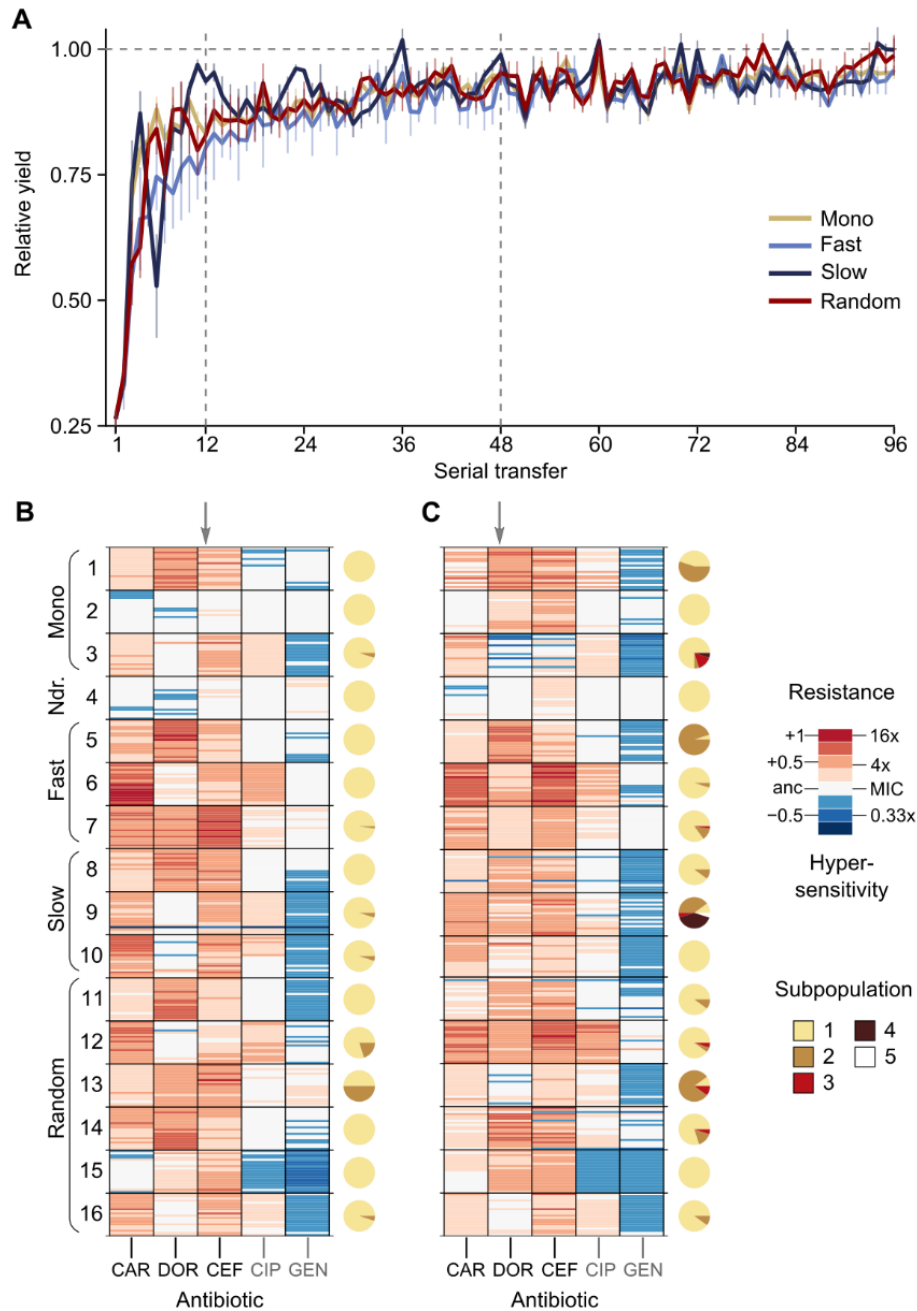
Extinction of experimental populations differed considerably between the antibiotic sets. The two  $\beta$ -lactam sets produced a surprisingly high degree of extinction (CAR-CEF-DOR and TIC-AZL-CEZ; extinct fraction 27.2% and 13.3% respectively, Fig. 1C). The observed extinction frequency was comparable to that observed in the previous experiment with CAR-CIP-GEN (extinct fraction 15%, Fig. 1C). CIP-DOR-STR caused no extinction, indicating that extinction was not explained by applying heterogeneous sets of antibiotics. Within the  $\beta$ -lactam sequential treatments we observed that treatments that switched between antibiotics fast (every transfer) produced much higher extinction levels than those which switched slowly (every four transfers) or not at all (Fig. 1D). Most of the extinction events happened early in the experiment (Fig. 1E), indicating that the initial treatment steps are critical for adaptation of populations. We conclude that fast sequential  $\beta$ -lactam treatments showed a surprising ability to restrict bacterial adaptation. As this result was unexpected, we decided to research the mechanisms that constrain resistance emergence in  $\beta$ -lactam sequences. Given that the experiment involving CAR-CEF-DOR produced the highest fraction of extinct populations, we decided to focus further analyses on this set.



**Figure 1. Probability of evolutionary rescue depends on drug triplets and treatment type.** (A) The evaluated antibiotic combinations comprise different number of antibiotic targets. (B) The evaluated treatment protocols test the effects of switching rate, and temporal regularity. (C) A fraction of lineages is eradicated by the sub-lethal dosage sequential treatments. Lineage extinction is high for combinations of cell-wall targeting  $\beta$ -lactams. (D) Variation in extinction for the  $\beta$ -lactam combinations by treatment type (n=3-6 protocols per treatment type). (E) The distribution of evolutionary trajectories for Exp. 3 with CAR-DOR-CEF shows that the majority of extinction events occur within the first 12 serial transfers (n = 180 lineages). AZL: azlocillin, CAR: carbenicillin, CEF: cefsulodin, CEZ: ceftazidime, CIP: ciprofloxacin, DOR: doripenem, GEN: gentamicin, STR: streptomycin, TIC: ticarcillin. The following supplementary material is available for Fig. 1: Figure 1-Figure Supplement 1, Figure 1-Source Data 1, Figure 1-Figure Supplement 1-Source data 1, Figure 1-Supplementary Table 1.

## **Resistance to doripenem was constrained in both monotherapy and switching treatments in the CAR-CEF-DOR triple $\beta$ -lactam experiment**

The CAR-CEF-DOR triple  $\beta$ -lactam experiment was characterized in detail for changes in growth, evolved resistance, and whole genome sequences, in order to assess the selection dynamics involved. We calculated the relative growth yield (Materials and Methods) at the end of each season and found growth dynamics to be divided into three phases: an early phase of rapid adaptation (transfers 1-12), followed by a phase of gradual growth yield convergence (transfers 13-48), and a final plateau phase (transfers 49-96) (Fig 2A). We compared the main treatment types using general linear models (GLM) for each phase separately (this fulfils the model assumption of response linearity). The early phase dynamics were characterized by significantly decelerated adaptation dynamics of the fast-regular group compared with monotherapy and slow-regular (GLM, post-hoc test,  $p < 0.037$ , Figure 2-Supplementary Table 1), but not random treatments. The slow-regular treatment did not differ significantly from monotherapy or random treatments (GLM, post-hoc test,  $p = 0.469$ , Figure 2-Supplementary Table 1). In the subsequent phase, growth yields of the groups converged to a plateau of roughly 90% relative yield, indicating similar final levels of adaptation (the growth yields of main treatment groups showed no statistical differences in phases 2 and 3, Figure 2-Supplementary Table 1). Alternating between the  $\beta$ -lactams fast and in a regular order therefore significantly constrained the growth of the bacterial populations. Intriguingly, in these fast sequential treatments, bacterial growth in the seasons with DOR was lower than in the seasons with the other two antibiotics (Figure 2-Figure Supplement 1), indicating an evolutionary constraint associated with the antibiotic DOR.



**Figure 2. Resistance to doripenem is constrained in the CAR-CEF-DOR triple  $\beta$ -lactam experiment.** (A) Rapid adaptive increase of biomass yields relative to the untreated reference populations (mean  $\pm$  CI95;  $n=3-6$  protocols per treatment type and 12 biological replicates per sequence; extinct lineages excluded). Evolved changes in the susceptibility to the treatment antibiotics CAR, DOR, and CEF and the non-treatment antibiotics CIP and GEN after transfer 12 (B) or transfer 48 (C), evaluated with 20 isolates each for the 16 representative adapting populations at each time point. The evolution of resistance and hypersensitivity are indicated by red and blue colour, respectively (total of 640 isolates). Pie charts show phenotypic within-population diversity, where different colors indicate subpopulations inferred from hierarchical clustering of resistance phenotypes. The following supplementary material is available for Figure 2: Figure 2-Figure Supplement 1, Figure 2-Figure Supplement 2, Figure 2-Source Data 1, Figure 2-Figure Supplement 1-Source Data 1, Figure 2-Figure Supplement 2-Source Data 1, Figure 2-Supplementary Table 1, Figure 2-Supplementary Table 2, Figure 2-Supplementary Table 3, Figure 2-Supplementary Table 4.

To understand the dynamics of early adaptation in more detail, we measured the resistance profiles of 16 evolved populations after transfers 12 and 48 from the different antibiotic treatments (representing the end of phases one and two, respectively; Fig. 2B and C, Figure 2-Figure Supplement 2, Materials and Methods). We randomly sampled 20 bacterial colonies from each population and characterized their resistance profile by broth microdilution. Resistance was measured for the three antibiotics of the evolution experiment and two additional clinically relevant antibiotics from different classes, ciprofloxacin and gentamicin. The resistance profiles in the early and the mid phases were found to be distinctly different. The middle phase (Fig. 2C) had increased  $\beta$ -lactam resistance (sum of resistances on CAR, CEF and DOR) if compared to the early phase (Fig. 2B), which was in line with the differences in growth dynamics between the two. We assessed how the main treatment types varied in their  $\beta$ -lactam resistance using a GLM for each phase separately. Most treatment types varied significantly from each other in their multidrug  $\beta$ -lactam resistance in both phases (Figure 2-Supplementary Table 2, Figure 2-Supplementary Table 3). The multidrug resistance in the early phase was in most cases constrained by the susceptibility to DOR (e.g., in the switching and monotherapy treatments). We observed collateral responses of the treatment to the two non- $\beta$ -lactams, which increased over time. We further used hierarchical clustering of the resistance profiles to assess the presence of sub-populations, followed by calculation of Shannon diversity for each population at both transfers. We found population diversity to be significantly higher at transfer 48 as compared to transfer 12 (ANOVA,  $F = 6.2060$ ,  $p = 0.01893$ , Figure 2-Supplementary Table 4), indicating a diversification of the evolving lineages over time. Taken together, the population analysis of resistance profiles indicates that the dynamics of resistance emergence to DOR is likely key for the observed deceleration of  $\beta$ -lactam adaptation in the fast-regular treatments.

To identify the genomic changes underlying the first steps of  $\beta$ -lactam adaptation we sequenced 33 whole genomes of the evolved and characterized isolates from the monotherapy, fast-regular, and slow-regular treatment types. Specifically, we sequenced three isolates from each population representing the distinct phenotypic sub-populations, assessed above. We found that all isolates, except those which received DOR monotherapy, had mutations in known resistance genes by the end of the early phase (Table 1). This agreed with the inferred resistance profiles where isolates from the DOR monotherapy did not show a noticeable amount of resistance at that stage (Fig. 2B). DOR resistance was, however, found at the end of the middle phase (Fig. 2C) and this was mirrored in the genomics with a non-synonymous mutation in the gene *ftsI*. This gene codes for the penicillin binding protein 3 (PBP3) (Liao and Hancock, 1995), a common target of all the three  $\beta$ -lactams (Davies et al., 2008; Fontana et al., 2000; Rodríguez-Tebár et al., 1982a; Rodríguez-Tebar et al., 1982b; Zimmermann, 1980). *ftsI* was also found to be mutated in isolates from CAR monotherapy, although at a different site within the gene and associated with a different resistance profile than the DOR-associated *ftsI* variant (Fig. 2B). Isolates from CEF monotherapy contained mutations in *pepA*. This gene is responsible for the production of a protein required for cytotoxicity and virulence in *P. aeruginosa* (Hauser et al., 1998). Although its role in antimicrobial resistance remains to be studied in detail, it was found to be mutated in *P. aeruginosa* strains resistant to certain  $\beta$ -lactams (Cabot et al., 2018; Sanz-García et al., 2018). The switching treatments selected for



mutations in the above-listed and also in some additional genes. In particular, we identified mutations in *nalD* and *phoQ*, a negative regulator of the MexAB-OprM efflux pump and a two-component system, respectively. Mutations in these genes account for resistance to a variety of drugs in *P. aeruginosa* (Barbosa et al., 2021; Sobel et al., 2005). Further mutations were identified in some non-canonical  $\beta$ -lactam resistance genes such as *rmcA*, 23srRNA, *3-oxoacyl synthase*, *dnaX* and *zipA* (Table 1). Taken together, mutations in both canonical and non-canonical targets of  $\beta$ -lactam selection were identified in our experiment, and among these, DOR resistance mutations only arose later in the experiment.

Based on our detailed characterization of the CAR-CEF-DOR triple  $\beta$ -lactam experiment, we conclude that DOR has a key role in restricting evolutionary rescue as evidenced by the delayed acquisition of genetic resistance to it.

**Table 1. Evolved genetic changes inferred from whole genome sequencing.**

Treatment type	ID <sup>a</sup>	AA change <sup>b</sup>	Gene name	Annotation	Freq <sup>c</sup>
Monotherapy	1	V471G	<i>ftsI</i>	Peptidoglycan synthesis	3/3
	2 <sup>d</sup>	N242S	<i>ftsI</i>	Peptidoglycan synthesis	3/3
	3	T157P	<i>pepA</i>	Virulence	3/3
Fast Regular	5	V471G	<i>ftsI</i>	Peptidoglycan synthesis	3/3
	6	K26	<i>nalD</i>	Efflux	3/3
		S379ISR	<i>rmcA</i>	Biofilm maintenance	1/3
	7	R220C	<i>phoQ</i>	Two-component	3/3
		-	23srRNA	Translation	1/3
Slow Regular	8	V471G	<i>ftsI</i>	Peptidoglycan synthesis	3/3
	9	D357N	<i>pepA</i>	Virulence	3/3
	10	T157P	<i>pepA</i>	Virulence	3/3
		E115VAAWIPK	3-oxoacyl-ACP synthase	Lipid metabolism	1/3
		Q117AEEQ	3-oxoacyl-ACP synthase	Lipid metabolism	1/3
		R178C	<i>zipA</i>	Cell division	2/3
		P483PEP	<i>dnaX</i>	Cell division	1/3

<sup>a</sup> Individual treatment of evolution experiment

<sup>b</sup> Amino acid change

<sup>c</sup> Occurrence frequency of the identified variant (before slash) out of the total number of isolates sequenced (behind slash) (*continued on the next page*)

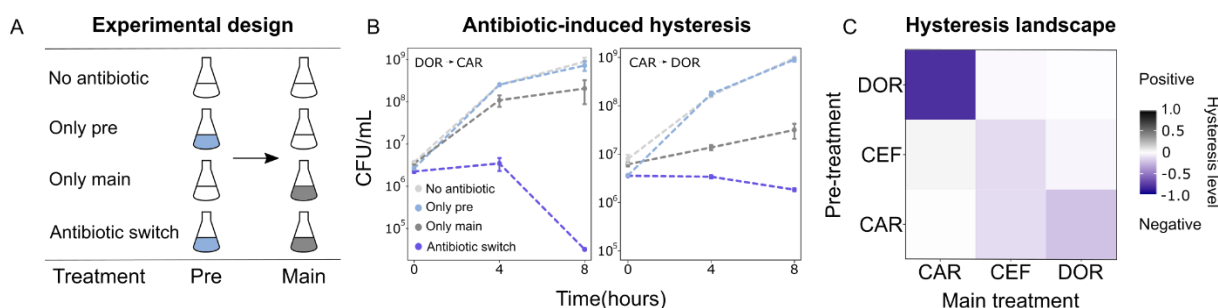
(continued from the previous page)

<sup>d</sup> Mutations listed are from populations frozen at season 48, no variants were found in populations frozen at season 12.

The following supplementary material is available for Table 1: Table 1-Source Data 1.

### Asymmetric bidirectional hysteresis was identified between doripenem and carbenicillin

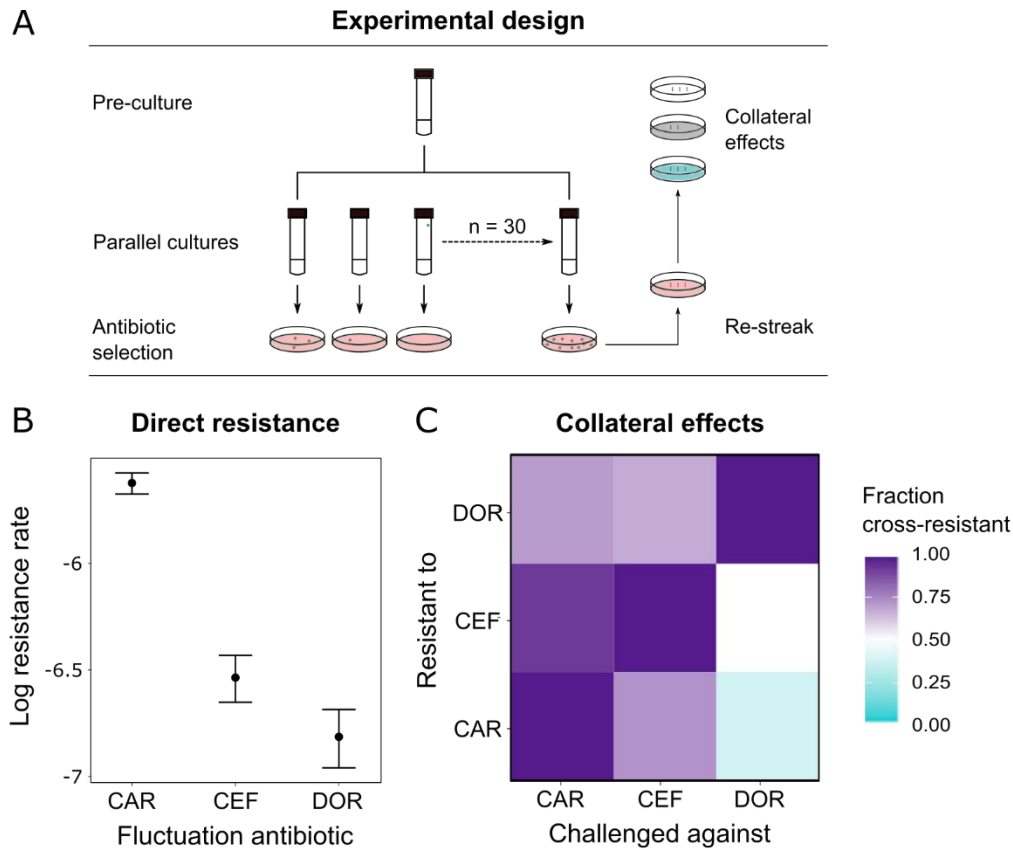
As extinction was associated to antibiotic switches, we next focused on selective events that can occur at drug switches, such as hysteresis, an inducible physiological change. We characterized the complete hysteresis landscape between the three  $\beta$ -lactams: CAR, DOR, and CEF. We pretreated exponential phase cells with an antibiotic for 15 min, followed by a change to fresh medium containing a second antibiotic as main treatment. We included controls of no pretreatment, or no main treatment (Fig. 3A). We found that negative hysteresis existed for several switches between the  $\beta$ -lactams (Figs. 3B and 3C, Figure 3-Figure Supplement 1). DOR and CAR displayed asymmetric bidirectional negative hysteresis with the switch from DOR to CAR resulting in more negative hysteresis than the reverse. Negative hysteresis was also observed in the switch from CAR to CEF and CEF to CEF. To our surprise, no positive hysteresis was observed, although we anticipated it given that *P. aeruginosa* produces the AmpC  $\beta$ -lactamase (Livermore, 1995). We conclude that negative hysteresis is abundant between the studied  $\beta$ -lactams and is a potential predictor of treatment potency in the sequential  $\beta$ -lactam treatments.



**Figure 3. Negative hysteresis is common among the tested  $\beta$ -lactam antibiotics.** (A) Hysteresis effects were measured using the previously established experimental approach (Materials and Methods). (B) Bacterial counts were plotted over time after the pre-treatment to obtain time-kill curves (mean  $\pm$  sem, n=3). Level of hysteresis was quantified as the difference between the antibiotic switch and the only main curves. Negative values indicate negative hysteresis and positive values indicate positive hysteresis (C) Heatmap of hysteresis levels between all 9 combinations of the three  $\beta$ -lactams. DOR and CAR show asymmetric bidirectional negative hysteresis. Negative hysteresis is also observed in switches from CEF to CEF and CAR to CEF. The following supplementary material is available for Figure 3: Figure 3-Figure Supplement 1, Figure 3-Source Data 1, Figure 3-Figure Supplement 1-Source Data 1.

### **Probability of direct and indirect resistance was the least for doripenem**

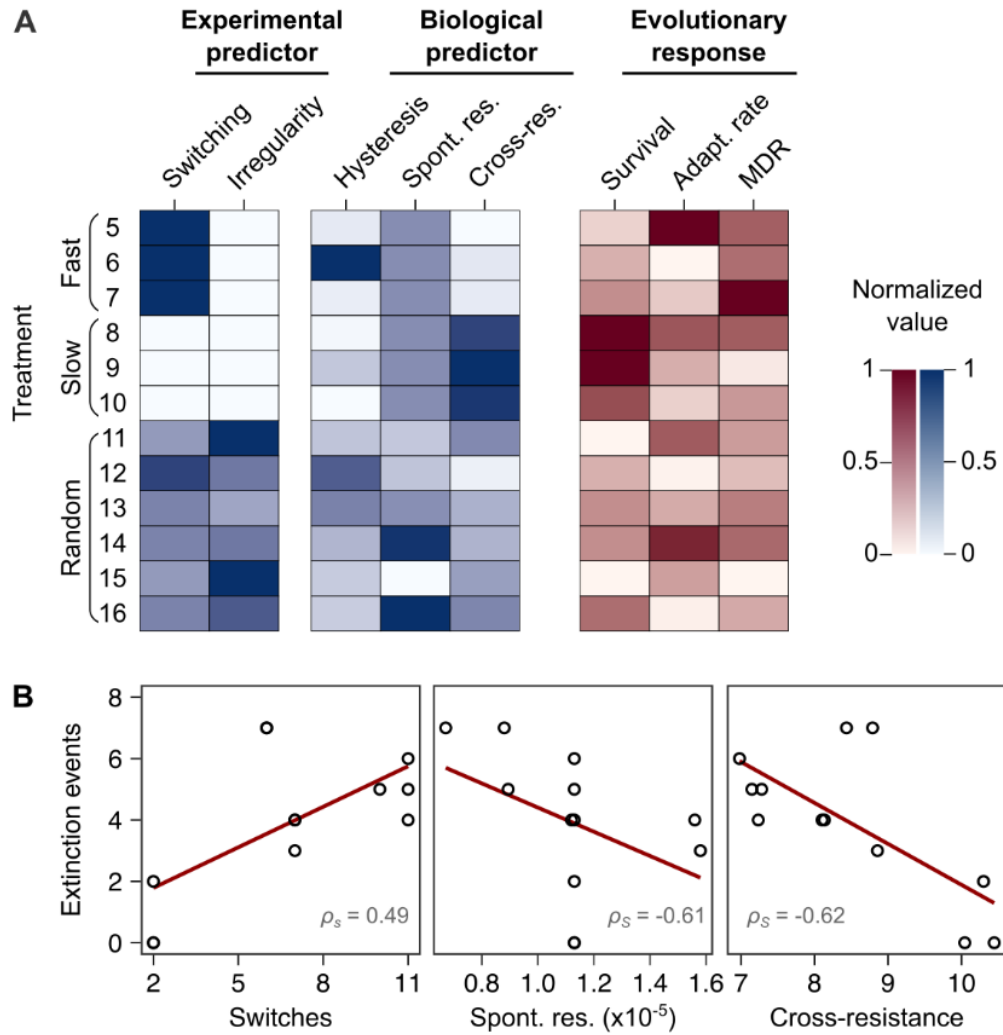
Since resistance to DOR was constrained in both the monotherapy and the switching treatments (Fig. 2B), we hypothesized that DOR resistance was difficult to achieve compared to the other two  $\beta$ -lactams. Resistance against a given drug can arise because of spontaneous direct resistance and/or because of collateral resistance from the preceding antibiotics in the sequence. As a first step, we thus measured the spontaneous direct resistance rate with the classic fluctuation assay (Luria and Delbrück, 1943, Fig. 4A, Figure 4-Supplementary Table 1). To determine the probability of indirect resistance in a second step, we isolated the obtained single-step mutants and quantified the fraction of cross-resistance towards the other two  $\beta$ -lactams with a patching assay (Fig. 4A). We used a comparatively large number of spontaneous mutants for this analysis ( $n = 60$  per antibiotic), in order to increase our ability to capture the stochastic nature of evolution and, in this context, the potential importance of collateral effects for bacterial adaptation, as previously emphasized (Nichol et al., 2019). We found that the spontaneous resistance rate was significantly lower for DOR than for CAR and CEF (Likelihood ratio test,  $p < 0.0001$  and  $p < 0.01$ , respectively; Figure 4-Supplementary Table 2, Fig. 4B). Moreover, the resulting cross-resistance effects (Fig. 4C) were particularly common towards CAR (93% of clones with spontaneous CEF resistance and 71% with DOR resistance) and CEF (73% of originally CAR-resistant clones and 67% DOR-resistant clones). By contrast, the smallest levels of cross-resistance were expressed towards DOR (36% of originally CAR-resistant clones and 50% CEF-resistant clones). The overall fraction of cross-resistant clones was significantly smaller towards DOR than either CEF or CAR (Fisher exact test,  $p = 0.0004$ ; Figure 4-Supplementary Table 3). We conclude that of the three  $\beta$ -lactams, DOR had the lowest probability for both direct and indirect resistance.



**Figure 4. Doripenem has the lowest rates of direct and indirect resistance.** (A) Schematic of the experimental protocol to determine spontaneous rates of resistance on each of the three  $\beta$ -lactams and the resulting collateral landscape. Briefly, an overnight culture was taken and split into 30 parallel cultures where bacteria were allowed to divide in the absence of an antibiotic and any other constraint. Spontaneous resistant mutants were selected on MIC plates and re-streaked to ensure genetic resistance. These mutants were then patched on MIC plates of the other two  $\beta$ -lactams to test for cross-resistance. (B) Comparison of rates of spontaneous resistance on the three  $\beta$ -lactams. Error bars depict CI95. All comparisons were found to be significantly different from each other (Likelihood Ratio Test; CAR vs CEF  $p < 0.0001$ , CAR vs DOR  $p < 0.0001$  and DOR vs CEF  $p < 0.01$ ). (C) Landscape of collateral effects between the three  $\beta$ -lactams. Fraction of cross-resistant mutants per antibiotic combination is plotted. Doripenem has the least cases of cross-resistance of the three. A total of 60 mutants per antibiotic were used for collateral effect testing. The following supplementary material is available for Figure 4: Figure 4-Source Data 1, Figure 4-Supplementary Table 1, Figure 4-Supplementary Table 2, Figure 4-Supplementary Table 3.

## **The rate of spontaneous resistance and resulting cross-resistance determine treatment efficacy**

We used the collected information to identify the critical determinant(s) of treatment efficacy in the CAR-CEF-DOR triple  $\beta$ -lactam experiment. We assessed the influence of either the two experimental predictors (switching rate, temporal irregularity) or the three biological predictors (hysteresis, probability of spontaneous resistance and resulting cross-resistance) on each of the evolutionary responses extinction, rate of growth adaptation, and multidrug resistance, using separate GLM-based analyses (Materials and Methods; Figure 5-Supplementary Tables 1-6). For the biological predictors, we calculated the levels of cumulative hysteresis, cumulative probability of spontaneous resistance, and the cumulative levels of cross-resistance in each of the 16 individual treatments up to season 12 (Materials and Methods). We focused our analysis on the early phase of evolution up to season 12, as it appeared most critical for treatment efficacy, especially for population extinctions that usually occurred early (Fig. 1E). Our analysis revealed that extinction was significantly associated with both the experimental predictors, switching rate (GLM,  $F=14.44$ ,  $p=0.0042$ , Fig. 5B, Figure 5-Supplementary Tables 1-4) and temporal irregularity (GLM,  $F=10.53$ ,  $p=0.0101$ , Figure 5-Supplementary Table 4). Temporal irregularity further showed a statistical trend with multidrug resistance (GLM,  $F=4.19$ ,  $p=0.0711$ , Figure 5-Supplementary Table 4). From our biological predictors, the cumulative cross-resistant fraction showed a significant association with extinction (GLM,  $F=10.42$ ,  $p=0.0121$ , Figure 5-Supplementary Table 6), while cumulative probability of spontaneous resistance showed a statistical trend (GLM,  $F=4.14$ ,  $p=0.0763$ , Figure 5-Supplementary Table 6). Indeed, the cumulative cross-resistant fraction and also the cumulative probability of spontaneous resistance are strongly correlated with extinction (Fig. 5B). The cumulative cross-resistant fraction is also strongly correlated with switching rate (Figure 5-Figure Supplement 1), most likely explaining the latter impact on extinction. By contrast, cumulative hysteresis levels did not have a significant influence on any of the evolutionary responses (GLM,  $F=0.16$ ,  $p=0.7015$ , Figure 5-Supplementary Table 6). Taken together, our results suggest that in our sequential CAR-CEF-DOR treatments the switching rate, temporal irregularity of antibiotics, the probability of spontaneous resistance, and especially the resulting collateral effects (maximized by switching rate) determine treatment efficacy through their effect on bacterial extinction. The limiting factor appears to be constrained evolution of resistance and low levels of cross-resistance to DOR.



**Figure 5. Bacterial extinction is correlated to switching rate, spontaneous rate of resistance and spontaneous cross-resistance.** (A) Variation in experimental parameters, potential biological predictors, and the measured traits up to transfer 12. The experimental parameters include switching rate, and regularity of change (high irregularity in dark). Potential biological predictors are cumulative levels of hysteresis (dark indicates protective effects), cumulative probabilities of spontaneous resistance (Spont. res., dark indicates higher probability), and cumulative level of collateral effects (Cross-res., dark indicates high fraction of cross-resistance). The evolutionary response was measured for population survival (max=12), adaptation rate (Adapt. rate,  $n \leq 12$ , extinct lineages excluded), evolved multidrug resistance to treatment antibiotics CAR, DOR, and CEF (MDR,  $n=16$ ). (B) Variation in extinction was best explained by collateral effects between the antibiotics (for illustrative purposes, the red line depicts linear regression and  $\rho_s$  the Spearman's rank correlation coefficient). The following supplementary material is available for Figure 5: Figure 5-Figure Supplement 1, Figure 5-Source Data 1, Figure 5-Supplementary Tables 1-6.

## Discussion

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Treatment with multiple  $\beta$ -lactam antibiotics is generally avoided, due to the perceived fear of therapy failure from cross-resistance. Our work now challenges this wide-spread belief. We characterized the ability of replicate *P. aeruginosa* populations to evolve *de-novo* resistance to sequential treatments with different drug sets. To our surprise, we found that sets of three  $\beta$ -lactams constrained bacterial adaptation by reducing bacterial survival. We demonstrate that treatment potency was determined by the spontaneous rate of resistance to the  $\beta$ -lactams and resulting cross-resistances between them during the sequential treatment protocols.

Our initial screen of sequential protocols with different antibiotic triplets revealed that the triple  $\beta$ -lactam sequences are at least as effective at causing extinction as sequences of antibiotics with distinct modes of actions. This finding is at first sight counter-intuitive, but at second sight not completely unexpected. The joint application of two  $\beta$ -lactam drugs was in fact tested and found effective in a few previous studies (Rahme et al., 2014). For example, the  $\beta$ -lactam aztreonam was shown to interact synergistically with four other  $\beta$ -lactam drugs against multiple resistant isolates of *Enterobacteriaceae* and *P. aeruginosa* *in vitro* (Buesing and Jorgensen, 1983). A combination of ticarcillin with ceftazidime produced high efficiency in a rat peritonitis model (Shyu et al., 1987). In a treatment of bacterial soft tissue infections, the combination of cefotaxin and mecillinam led to higher clinical response rates than the tested monotherapy (File and Tan, 1983). Further, the dual  $\beta$ -lactam combination of ceftazidime plus piperacillin was as effective as the combination of ceftazidime and tobramycin in granulocytopenic cancer patients (Joshi et al., 1993). More recent studies demonstrated that a triple combination of meropenem, piperacillin and tazobactam successfully constrained resistance evolution in Methicillin-resistant *Staphylococcus aureus* (MRSA), both *in vitro* and in a mouse model (Gonzales et al., 2015). In addition, the combination of cefotaxime and mecillinam was effective against *Salmonella enterica* harbouring a mutant  $\beta$ -lactamase in a mouse model (Rosenkilde et al., 2019). Our findings add to the high potency of treatments with multiple  $\beta$ -lactams. We conclude that the use of multiple  $\beta$ -lactams, either as a combination or sequentially, is a commonly underappreciated form of therapy and its use opens new avenues to better utilize our existing antibiotic armamentarium.

Spontaneous rate of antibiotic resistance was found to play a critical role in the success of the CAR-CEF-DOR sequential treatment. The probability of spontaneous resistance on all three  $\beta$ -lactams was significantly different, with the rate of DOR resistance being the lowest. These rates determined the overall probability of acquiring direct resistance in treatment, which significantly correlated with the frequency of population extinction (Fig. 5B). Resistance rates were previously shown to vary towards different antibiotics, for example in *E. coli* (Meouche and Dunlop, 2018) and *P. aeruginosa* (Oliver et al., 2004). This variation can arise from genetic factors such as mutational target space and physiological factors like activation of the bacterial SOS response (Martinez and Baquero, 2000). Such information on resistance rates have so far been used for predicting the occurrence of resistance against single drugs, and antibiotics that target multiple pathways in a cell are considered advantageous in this context (Ross-Gillespie and Kümmerli, 2014). One example of the latter are compounds against *S. aureus* that inhibit both, DNA gyrase and Topoisomerase IV (Nyerges et al., 2020). The rate of resistance

emergence may also be reduced by using adjuvants that target the SOS response (Bell and MacLean, 2018), as previously shown for compounds interfering with LexA activity leading to reduced resistance rates to ciprofloxacin and rifampicin in *E. coli* (Cirz et al., 2005). Our study extends the role of resistance rates of antibiotics beyond this convention. We show that inclusion of an antibiotic with relatively low spontaneous resistance emergence can enhance the potency of a sequential treatment design.

A key determinant of treatment potency was the reduced level of spontaneous cross-resistance to the sequentially applied drugs (Fig. 5B). This effect was maximized by the switching rate (Figure 5-Figure Supplement 1). Our findings are consistent with the previously and repeatedly proposed importance of collateral sensitivity for the efficacy of sequential treatment protocols (Barbosa et al., 2019; Hernando-Amado et al., 2020; Imamovic and Sommer, 2013; Kim et al., 2014; Maltas and Wood, 2019; Yen and Papin, 2017). Even though we did not measure collateral sensitivity directly, the lack of cross-resistance is related, as it indicates that the mutant cells, which have become resistant to one drug, maintain at least ancestral levels of susceptibility against the second drug. Moreover, our study focused on spontaneous emergence of cross-resistance (or lack thereof). By contrast, many previous studies established collateral effects after bacteria evolved resistance to the first drug over many generations, often followed by only a single antibiotic switch to assess the impact of collateral sensitivity on therapy success (Barbosa et al., 2019; Hernando-Amado et al., 2020; Imamovic and Sommer, 2013; Yen and Papin, 2017). Surprisingly, our study revealed potentially beneficial collateral effects between antibiotics of the same class. In fact, we chose these three  $\beta$ -lactams because our previous work demonstrated disadvantageous cross-resistance between most of them, although inferred upon multigenerational adaptation to the first drug (Barbosa et al., 2017). Our current finding of a lack of cross-resistance among some of these drugs now suggests that spontaneous mutants may have different collateral profiles than the lines, which adapted over many generations. Our results further suggest that the collateral effects of spontaneous mutants are likely to be more pertinent for the design of sequential treatments with fast switches among antibiotics. This suggestion is supported by two previous studies, in which the efficacy of fast sequential treatments was optimized by considering collateral effects for either single-step mutants of *Staphylococcus aureus*, obtained after 20 h exposure to three distinct antibiotics for 20 h (Kim et al., 2014), or from *Enterococcus faecalis* populations adapted over two days to four distinct antibiotics (Maltas and Wood, 2019). As a side-note, it is particularly interesting that our detailed resistance analysis consistently revealed almost all treatments to cause the evolution of collateral sensitivity towards the aminoglycoside gentamicin, but not the fluoroquinolone ciprofloxacin (Fig. 2B, 2C), possibly indicating yet another treatment option – in cases where the applied triple  $\beta$ -lactam sequential protocols fail.

Temporal irregularity was additionally found to constrain bacterial adaptation. When bacteria experienced the antibiotics in an irregular pattern, this caused significantly increased extinction and to some degree reduced multidrug resistance. Environmental change anticipation has been documented in several microorganisms (Mitchell et al., 2009; Mitchell and Pilpel, 2011), indicating their capability to specifically adapt to regular environmental change. Stochastic changes can make it harder to evolve anticipation (Roemhild and Schulenburg, 2019). Stochastic changes in environmental parameters were indeed found to



constrain fitness in evolving bacteria (Hughes et al., 2007) and viruses (Alto et al., 2013). We show that stochastic changes, even between similar antibiotics, can be important to counter bacterial resistance evolution.

Unexpectedly, we further identified negative hysteresis for multiple combinations of the three  $\beta$ -lactams. However, cumulative hysteresis levels per treatment did not significantly associate with any of our measured evolutionary responses. In our previous study (Roemhild et al., 2018), within the CAR-CIP-GEN combination, negative hysteresis was expressed for the switches from CAR to GEN and CIP to GEN. Yet, only the CAR-GEN hysteresis was significantly associated to the evolutionary responses. We conclude that hysteresis interactions can exist between antibiotics from the same or different classes, but they need not impact the evolutionary outcome of a sequential treatment protocol each time. Nevertheless, it clearly warrants further research to assess whether negative hysteresis between the  $\beta$ -lactam drugs can somehow be exploited in sequential therapy, in analogy to the previous results with antibiotics from different classes (Roemhild et al., 2018).

Taken together, our study highlights that the available antibiotics offer unexplored, highly potent treatment options that can be harnessed to counter the spread of drug resistance. Ironically, the differential cross-resistance landscape of the  $\beta$ -lactams was a key factor contributing to treatment potency, even though the risk of cross-resistance is usually used to reject  $\beta$ -lactam exclusive treatments. The underlying reasons for reduced spontaneous cross-resistance between these drugs (including the underlying molecular mechanisms) are as yet unknown and clearly deserve further attention in the future. We conclude that a detailed understanding of both spontaneous resistance rates and resulting cross-resistances against different antibiotics should be of particular value to further improve the potency of sequential protocols.

## Materials and methods

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### Materials

All experiments were performed with *P. aeruginosa* UCBPP-PA14 (Rahme et al., 1995). Bacteria were grown in M9 minimal medium supplemented with glucose (2 g/L), citrate (0.58 g/L) and casamino acids (1 g/L) or on M9 minimal agar (1.5%) or Luria-Bertani (LB) Agar. Antibiotics were added as indicated. Cultures and plates were incubated at 37°C. Experiments included biological replicates (initiated with independent clones of the bacteria, which were grown separately before the start of the experiment, or independent evolutionary lineages from the respective evolution treatments) and technical replicates (initiated from the same starting culture of the bacteria), as indicated below. For the experiments, treatment groups were run in parallel and randomized. Treatment names were masked, in order to minimize observer bias.

### Dose-response curves of ancestor

We used dose-response curves based on broth microdilution, in order to determine antibiotic concentration causing inhibition level of 25% growth yield relative of untreated controls (inhibitory concentration 75; IC75) for the antibiotics azlocillin (AZL), carbenicillin (CAR),

ciprofloxacin (CIP), cefsulodin (CEF), ceftazidime (CTZ), doripenem (DOR), gentamicin (GEN) and ticarcillin (TIC; see Figure 1-Supplementary Table 1 for details on antibiotics). Briefly, bacteria were grown to exponential phase ( $OD_{600} = 0.08$ ) and inoculated into 96-well plates (100  $\mu$ l per well,  $5 \times 10^6$  CFU/ml) containing linear concentration ranges close to minimum inhibitory concentration of the antibiotics in M9 medium. Antibiotic concentrations were randomized spatially. Bacteria were incubated for 12 hours after which optical density was measured in BioTek EON plate readers at 600 nm ( $OD_{600}$ ). We included 6 biological replicates and 1-2 technical replicates per concentration and antibiotic. Optical density was plotted against antibiotic concentration to obtain a dose-response curve. Model fitting was carried out using the package *drc* (Ritz et al., 2015) in the statistical environment R and the fitted curve was used to predict IC75 values (Figure 1-Figure Supplement 1).

### **Evolution experiments**

We carried out evolution experiments with the various combination of antibiotics according to the design described previously (Roemhild et al., 2018). A total of 16 treatments were included (Fig. 1B). Treatments 1-4 were constant environments consisting of the monotherapy (#1-3) and no drug control (#4). Treatments 5-10 were the regular switching treatments. They switched between the antibiotics in a regular predictable fashion, either every transfer (fast; #5-7) or every fourth transfer (slow; #8-10). Treatments 11-16 consisted of the random treatments which switched fast in a temporally irregular fashion. The setup was designed to test the effect of switching rate and temporal irregularity.

Every treatment consisted of 12 replicate populations, 6 biological replicates with 2 technical replicates each. All populations were started with an inoculum of  $5 \times 10^5$  cells. Populations were propagated as 100  $\mu$ L batch cultures in 96-well plates, with a transfer to fresh medium every 12 hours (transfer size 2% v/v). Antibiotic selection was applied at IC75 throughout. We monitored growth by  $OD_{600}$  measurements taken every 15 min through the entire evolution experiment (BioTek Instruments, USA; Ref. EON; 37 °C, 180 rpm double-orbital shaking). Evolutionary growth dynamics were assessed by plotting the final OD achieved in every transfer (relative to final OD of no drug control; relative yield). Adaptation rate was calculated with a sliding window approach, where adaptation rate was the inverse of the transfer at which the mean relative yield of a sliding window of 12 transfers reached 0.75 for the first time. Cases of extinction were determined at the end of the experiment by counting wells in which no growth was observed after an additional incubation in antibiotic-free medium. Samples of the populations were frozen in regular intervals in 10% (v/v) DMSO and stored at -80 °C for later analysis. The evolution experiments were carried out for a total of 96 transfers.

### **Resistance measurements of evolved populations**

We characterized populations frozen at transfers 12 and 48 in detail, because they represented the early and late phases of the evolution experiment. One population, originating from a single biological replicate was chosen per treatment and plated onto LB Agar. After incubation at 37°C, 20 colonies from each population were picked randomly and frozen in 10% (v/v) DMSO and stored at -80°C. These colonies, termed isolates, were considered to be representative

biological replicates for each population. We constructed dose-response curves for the isolates, using for each evolved population 1 technical replicate per isolate and 4 technical replicates of the ancestral PA14 strain, as described above, for the antibiotics CAR, CEF, DOR, GEN and CIP. The integral of this curve for every isolate was calculated and the integral of the ancestral PA14 control subtracted. The resulting value was resistance of the isolate on the said antibiotic. We identified sub-populations in any given population by hierarchical clustering of the resistance profiles, as previously described (Roemhild et al., 2018). Resistance of a population was calculated by averaging the resistance of the isolates. Resistance of the population on CAR, CEF, and DOR were added to obtain a single value for multidrug resistance.

### **Whole-genome sequencing**

From the frozen isolates at transfer 12, we chose 3 isolates per population (i.e., three biological replicates per population) for whole genome sequencing to determine possible targets of selection. Each resistance cluster in the population was represented in the sequenced isolates. For the DOR monotherapy isolates from transfer 48 were also sequenced as no phenotypic resistance was observed at transfer 12. Frozen isolates were thawed and grown in M9 medium at 37 °C for 16-20 hours. We extracted DNA using a modified CTAB protocol (von der Schulenburg et al., 2001) and sequenced it at the Competence Centre for Genomic Analysis Kiel (CCGA Kiel; Institute for Clinical Microbiology, University Hospital Kiel), using Illumina Nextera DNA Flex library preparation and the MiSeq paired-end technology (2 x 300 bp). Quality control on the resulting raw reads was performed with FastQC (Andrews, 2010) and low quality reads were trimmed using Trimmomatic (Bolger et al., 2014). We then used MarkDuplicates from the Picard Toolkit (<http://broadinstitute.github.io/picard/>) to remove duplicate reads and mapped the remaining reads to the *P. aeruginosa* UCBPP-PA14 genome (available at <http://pseudomonas.com/strain/download>) using Bowtie2 and samtools (Langmead and Salzberg, 2012; Li et al., 2009). Variant calling was done using the GATK suite (Poplin et al., 2018) and the called variants were annotated using SnpEFF (Cingolani et al., 2012) and the Pseudomonas Genome Database ([www.pseudomonas.com](http://www.pseudomonas.com)). We removed all variants that were detected in the no drug control as they likely represent adaptation to the medium and not the antibiotic. The fasta files of all sequenced isolates are available from NCBI under the BioProject number: PRJNA704789.

### **Hysteresis testing**

The presence of cellular hysteresis was tested, following the previously developed protocol (Roemhild et al., 2018). Bacterial cells were grown to exponential phase ( $OD_{600} = 0.08$ ), diluted 10-fold and treated with IC75 of the first antibiotic. In the treatments where the pre-treatment did not require an antibiotic, none was added. These cells were allowed to incubate for 15 min at 37°C and 150 rpm (pre-treatment). After this, the first antibiotic was removed by centrifugation and fresh medium containing IC75 of a second antibiotic was added. In cases where the main treatment did not require an antibiotic, fresh medium without an antibiotic was added. Bacteria were now incubated for 8 hours at 37°C and 150 rpm (main treatment). Bacterial count was monitored through the main treatment by spotting assays. We used three biological replicates per treatment and, for CFU counting, four technical replicates per biological replicate and treatment.  $\log_{10}$  CFU/mL were plotted against time to obtain time-kill

curves (Fig. 3B). The level of hysteresis was calculated as the difference between the antibiotic switch and only main treatment curves.

### Agar dilution

We determined the minimum inhibitory concentration on M9 agar for the antibiotics CAR, CEF and DOR according to the EUCAST protocol (<https://doi.org/10.1046/j.1469-0691.2000.00142.x>) that was modified to account for inoculum effect in our fluctuation assay setup. UCBPP-PA14 was grown in M9 medium at 37°C for 20 hours.  $5 \times 10^5$  cells were taken from the stationary phase cultures and spread on M9 agar plates containing doubling dilutions of the antibiotic. Plates were incubated at 37°C for 20-24 hours. MIC was read as the lowest concentration at which no growth of bacteria was seen. MIC determination for each antibiotic was done for three biological replicates (no additional technical replication).

### Fluctuation assay

We measured resistance rates on the three  $\beta$ -lactams using the classic fluctuation assay (Luria and Delbrück, 1943). Briefly, a single colony of UCBPP-PA14 was inoculated to 10 mL M9 and incubated at 37°C, 150 rpm for 20 hours. This primary culture was used to start 30 parallel cultures all having a starting concentration of  $10^2$  CFU/mL. The parallel cultures were considered biological replicates and incubated at 37°C, 150 rpm for 20 hours. Thereafter,  $5 \times 10^5$  cells were plated onto MIC plates of CAR, CEF and DOR. The plates were incubated for 40 hours at 37°C. The resulting mutant colonies were taken and patched on identical antibiotic MIC plates to ensure genetic resistance. Colonies which grew after patching were counted. We used counts from all 30 cultures to estimate resistance rate on each antibiotic using the package *rSalvador* (Zheng, 2017) in R.

### Patching assay

We assessed the extent of cross-resistance associated with each  $\beta$ -lactam using the mutants obtained from the fluctuation assay. Sixty mutants with genetic resistance to a given  $\beta$ -lactam were considered biological replicates and patched onto MIC plates of the two other  $\beta$ -lactams. The patched plates were incubated for 16-20 hours at 37°C. If the mutant grew at MIC of the second  $\beta$ -lactam it was counted as resistant. If it did not grow at the MIC of the second  $\beta$ -lactam it was counted as susceptible. For each switch between two drugs, the fraction of cross-resistant mutants was calculated as

$$\frac{\text{Number of mutants that grew on drug B}}{\text{Total mutants isolated on drug A}}$$

### Statistical analysis for cross-resistance on secondary antibiotic

To test whether the secondary antibiotic had an influence on the degree of cross-resistance of the mutants obtained from the fluctuation assay we conducted a Fischer's exact test followed by posthoc comparisons using the R package *rcompanion* (Mangiafico, 2016). The obtained *p*-values were then corrected for multiple testing using *false-discovery rate*.

## Statistical analysis of adaptive growth dynamics

To test whether main treatment types were associated with altered dynamics of adaptation in non-extinct populations, we analyzed the trajectories of relative growth yield (as plotted in Fig 1E and Fig 2A) of drug-treated populations using a general linear model (GLM), including sequence (##1-16) and transfer as fixed factors and preculture and replicate population as nested random factors (see Figure 2-Supplementary Table 1 for details). Comparisons between main treatment groups were performed using pairwise post-hoc tests and  $z$  statistics. All  $p$ -values were corrected for multiple testing using *false-discovery rate*. The analysis was performed separately for the three time phases “early” (transfers 2-12), “middle” (transfers 13-48), and “late” (transfers 49-96) of the experiment, thus fulfilling the model assumption of response linearity. All statistical analyses were carried out in the statistical environment R.

## Statistical analysis of evolved multidrug $\beta$ -lactam resistance

To test whether evolved populations displayed distinct multidrug  $\beta$ -lactam resistance depending on their main treatment type, we analyzed multidrug  $\beta$ -lactam resistance of evolved isolates – the sum of resistance values against CAR, CEF, and DOR (as plotted in Fig 3BC) – using a general linear model (GLM). The model included sequence (##1-16) as fixed factor and replicate population as nested random factor (see Figure 2-Supplementary Table 2 for detailed information). Comparisons between main treatment groups were performed using pairwise post-hoc tests and  $z$  statistics. All  $p$ -values were corrected for multiple testing using *false-discovery rate*. The analysis was performed separately for the “early” (after transfers 12) and “middle” (transfer 48) time points of the evolution experiment, using the R statistical environment.

## Statistical analysis of treatment potency predictors

To test whether our experimental (switching rate and temporal irregularity) and biological predictors (hysteresis, probability of direct resistance and cross effects) were able to explain the variability in our evolutionary responses (extinction, rate of growth adaptation and multidrug resistance) we carried out a general linear model (GLM) analysis. Values per treatment protocol for the biological predictors were calculated and the GLM analysis then carried out in R. We used the *lm* and *anova* commands and the main effects model: response ~ switching rate + irregularity for the experimental predictors and response ~ hysteresis + spontaneous resistance + mutant fraction sensitive for the biological predictors.

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#### **Competing interests statement**

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The authors declare no competing interests.

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# HIGH POTENCY OF SEQUENTIAL THERAPY WITH ONLY $\beta$ -LACTAM ANTIBIOTICS

## SUPPLEMENTARY INFORMATION

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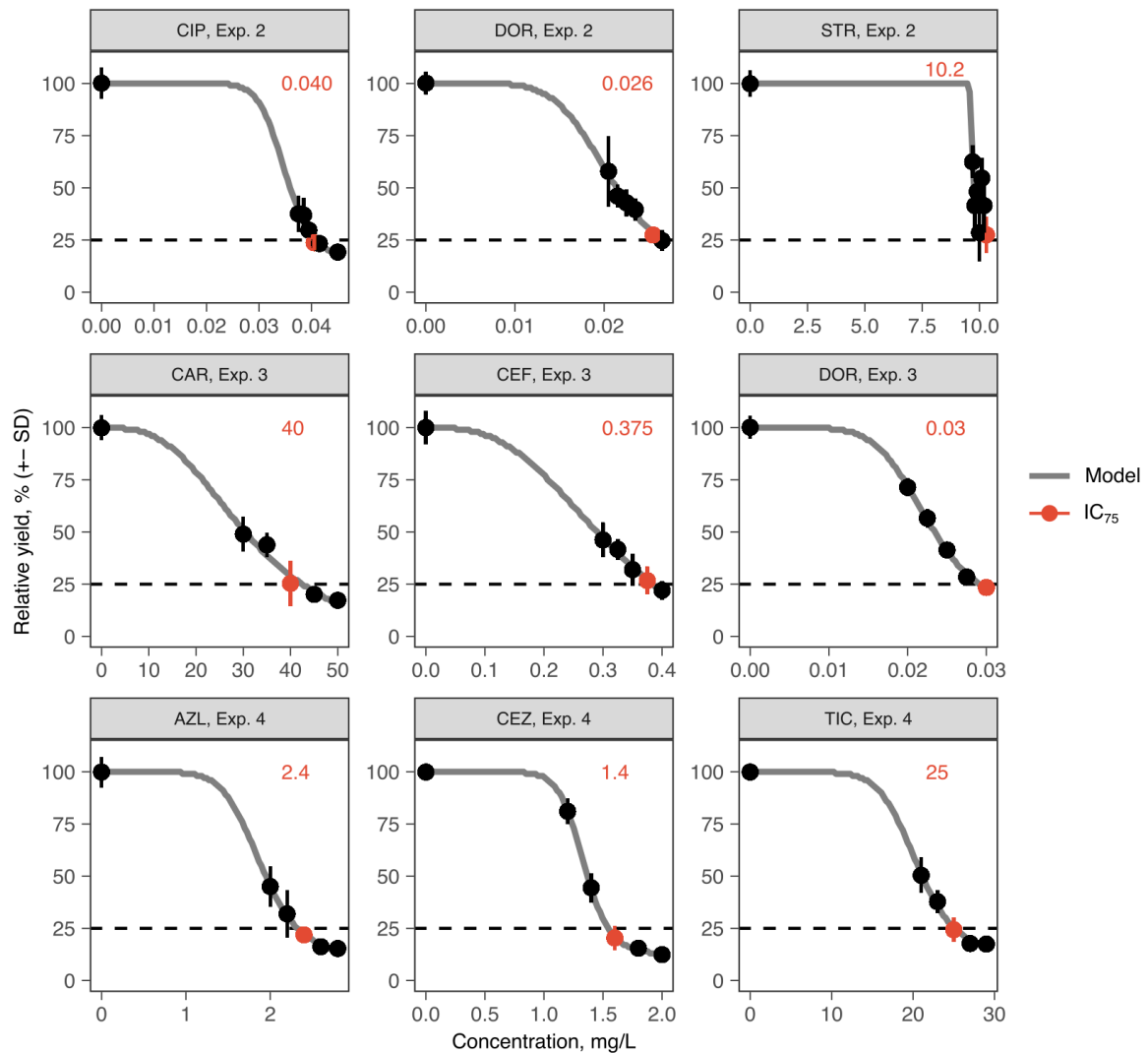
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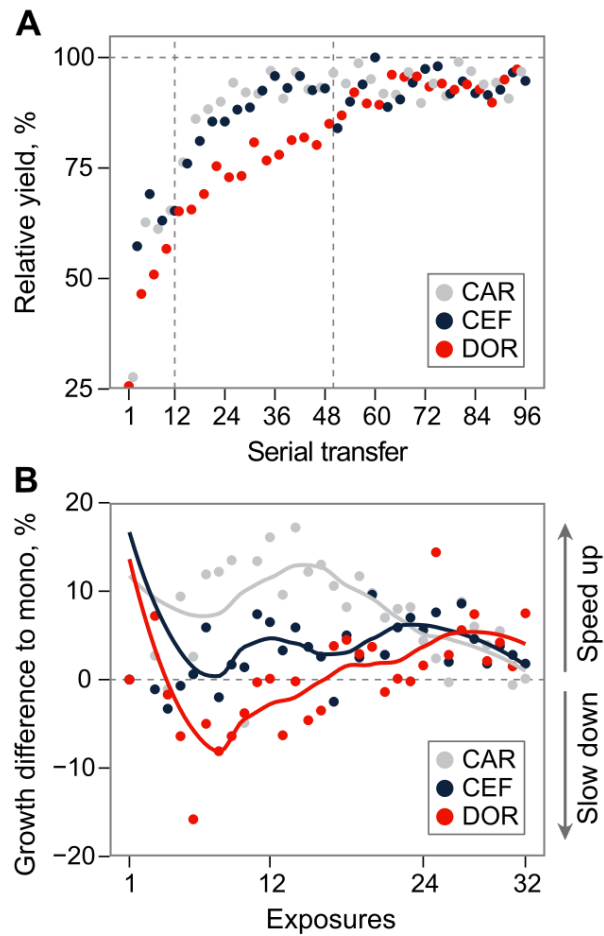
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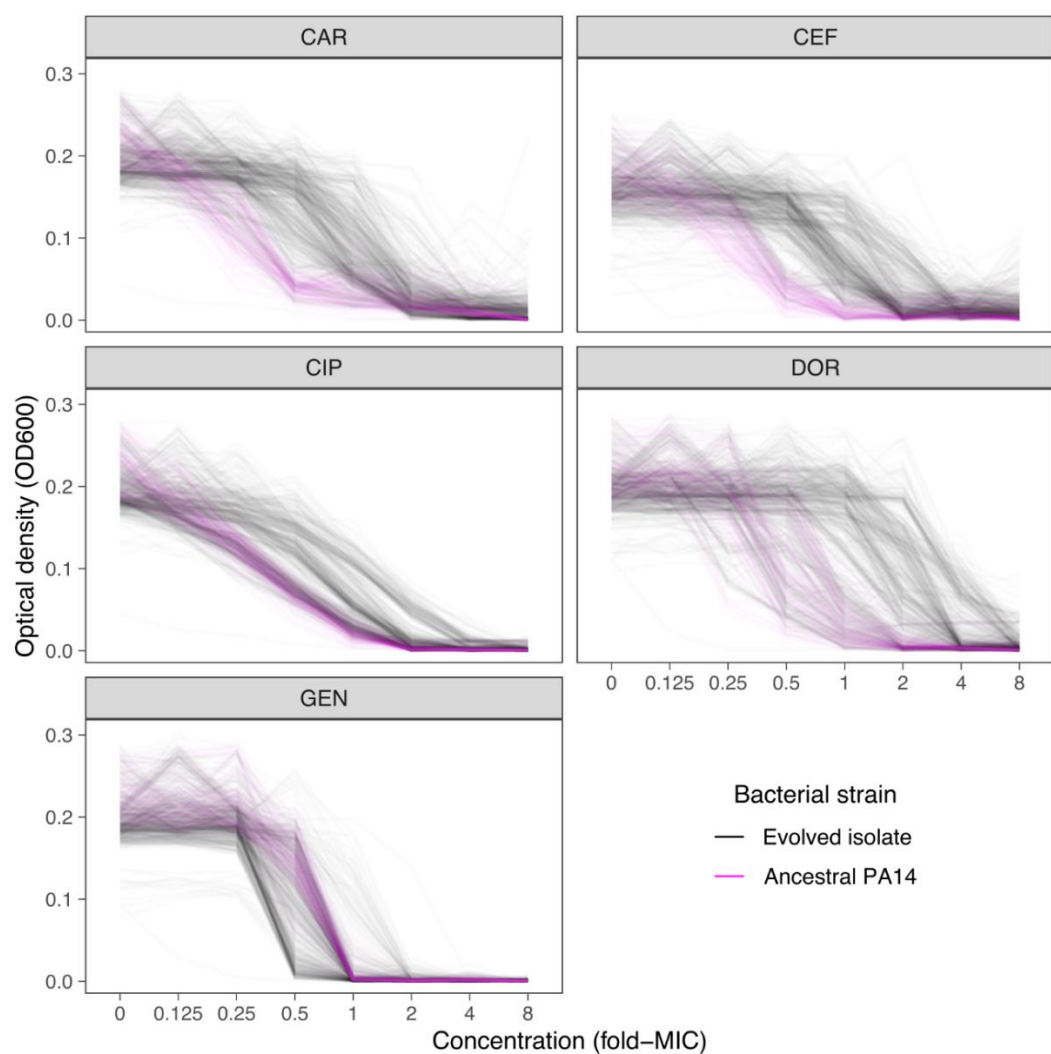
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**Figure 1-Figure Supplement 1.** Antibiotic dose-response curves for PA14 (mean  $\pm$  s.d.;  $n=6$  biological replicates). Red text and points indicate IC<sub>75</sub> inhibitory concentrations as applied in the evolution experiments. Grey line indicates Nelder-Mead dose-response model (*R*-package *drc*). The source data is provided in Figure 1-Figure Supplement 1-Source Data 1.

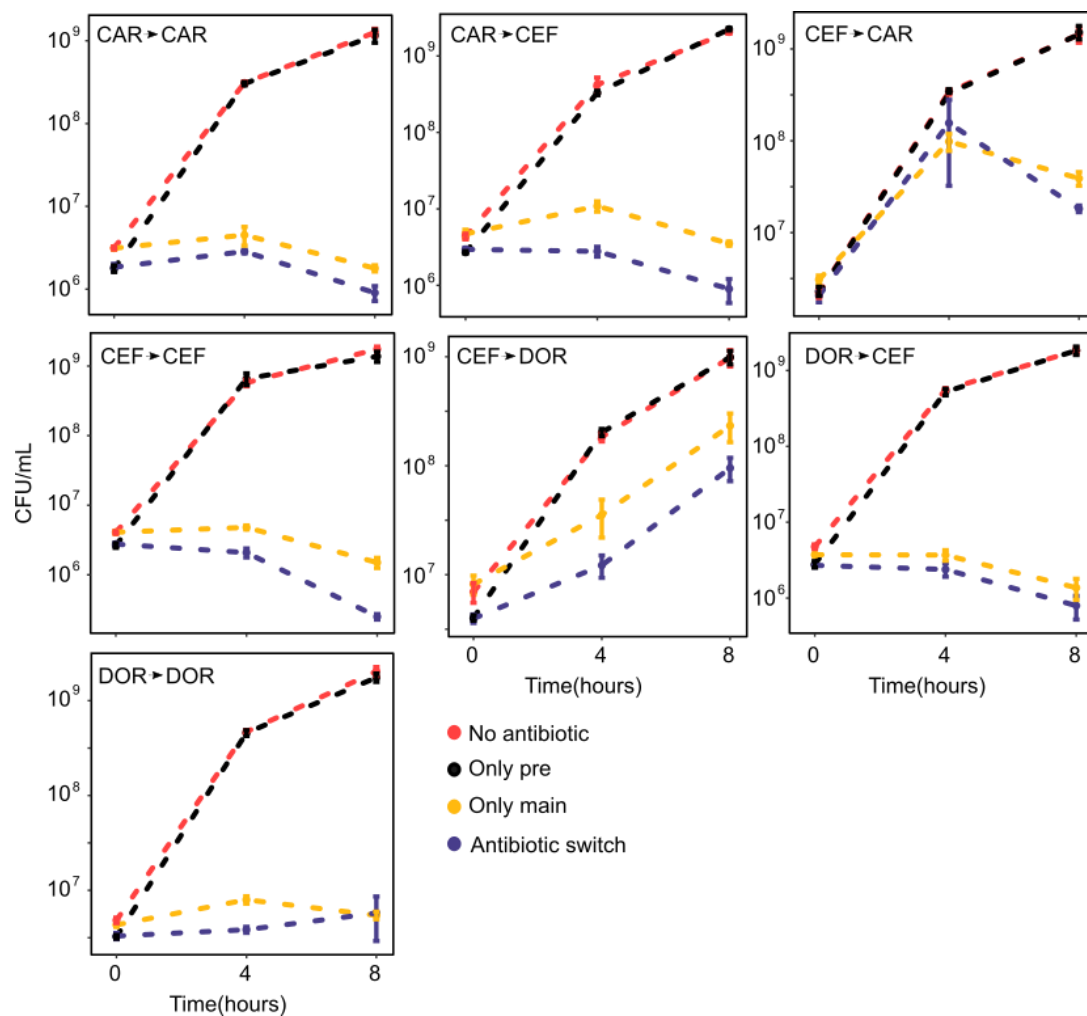


**Figure 2-Figure Supplement 1.** Growth dynamics in fast sequential protocols. (A) Evolutionary growth improvements for fast protocol #6 (mean of the 7 surviving lineages). Relative growth increased to the antibiotics at different rates, demonstrating the consecutive evolution of resistance, and thus coexistence of genetic subpopulations. The resulting clonal interference may explain the drop of growth on CEF around transfer 50, and the subsequent growth oscillations during CEF. (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 a particular antibiotic, and thus goes to  $96/3 = 32$ . Triple exposure accelerated adaptation compared to monotherapy against CAR and CEF, but slowed down adaptation against DOR. The source data is provided in Figure 2-Figure Supplement 1-Source Data 1

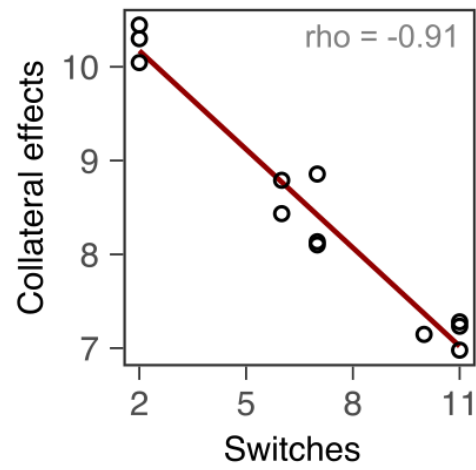


**Figure 2-Figure Supplement 2.** Dose-response curve distributions for Exp. 3 with CAR-DOR-CEF, underlying Fig 2B and Fig 2C. Grey lines show data from evolved isolates, magenta lines show repeated measurements of the PA14 ancestor. The source data is provided in Figure 2-Figure Supplement 2-Source Data 1.





**Figure 3-Figure Supplement 1.** Time-kill curves for quantifying hysteresis effects for the combinations not presented in Fig. 3 (mean  $\pm$  sem,  $n=3$ ). The source data is provided in Figure 3-Figure Supplement 1-Source Data 1.



**Figure 5-Figure Supplement 1.** Correlation of switching rate with cumulative levels of collateral effects.

**Figure 1-Supplementary Table 1. List of antibiotics used for the evolution experiments**

<b>Abbr.</b>	<b>Antibiotic</b>	<b>Supplier</b>	<b>Solvent</b>	<b>Stock, mg/ml</b>	<b>Powder , °C</b>
<b>AZL</b>	Azlocillin	Sigma, A7926-1G Prepare fresh	Water	50	4
<b>CAR</b>	Carbenicillin	Carl Roth, 6344.2 Prepare fresh	50% EtOH	50	4
<b>CIP</b>	Ciprofloxacin	Sigma, 17850-5G-F	0.1 M HCl	25	RT
<b>CEF</b>	Cefsulodin	Carl Roth, 4014.2 Light sensitive!	Water	20	-20
<b>CTZ</b>	Ceftazidime	Sigma, C3809.1G with 10% NaCO <sub>3</sub> renew at least monthly	Water (pH ~ 9.0) PBS (pH = 7.4)	11 (= 10 mg/ml CTZ)	4
<b>DOR</b>	Doripenem	Sigma, 32138-25MG	Water (37°C)	25	4
<b>GEN</b>	Gentamicin solution	Roth, 2475.1	Ready made	50	n.a.
<b>STR</b>	Streptomycin sulfate	Sigma, S6501-5	Water	25	4
<b>TIC</b>	Ticarcillin	Sigma, T5639-1G Prepare fresh	Water	100	4

**Figure 2-Supplementary Table 1. Statistical analysis of main evolution treatments for the evolutionary dynamics shown in Figure 2A<sup>a</sup>.**

Comparison	<i>z</i>	<i>P</i>
<b>Early phase (transfers 2-12)</b>		
mono-fast regular	3.16	<b>0.00947</b>
mono-slow regular	0.724	0.46897
mono-random	1.527	0.19012
fast regular-slow regular	-2.505	<b>0.03673</b>
fast regular-random	-1.948	0.10294
slow regular-random	0.76	0.46897
<b>Middle phase (transfers 13-48)</b>		
mono-fast regular	1.407	0.319
mono-slow regular	-0.779	0.523
mono-random	0.01	0.992
fast regular-slow regular	-2.061	0.236
fast regular-random	-1.444	0.319
slow regular-random	0.816	0.523
<b>Late phase (transfers 49-96)</b>		
mono-fast regular	0.162	0.871
mono-slow regular	-0.647	0.621
mono-random	-1.399	0.507
fast regular-slow regular	-0.723	0.621
fast regular-random	-1.375	0.507
slow regular-random	-0.685	0.621

<sup>a</sup> The analysis was performed with R version 3.5.0. Post-hoc pairwise comparisons based on *z* statistics, following analysis of a general linear mixed model (GLMM), including relative growth yield as the response variable and sequence and transfer as fixed factors and preculture and replicate population as nested random factors. Representation of the model using R package *nlme*: *lme(data=data\_noex, rgr ~ evoexp\_ID + season, random=list(~1/preculture, ~season/line))*. The defined model provided a better fit than a minimal model for all three phases (*Likelihood ratio*>254, *P*<0.0001). All significant *P* values are shown in bold (*P* values were corrected for multiple testing using *false discovery rate*). Extinct lineages excluded.

**Figure 2-Supplementary Table 2. Statistical analysis of main evolution treatments for the multidrug  $\beta$ -lactam resistance after transfer 12 in Fig 2B<sup>a</sup>.**

Comparison	<i>z</i>	<i>P</i>
<b>Early phase (transfer 12)</b>		
mono-fast regular	-11.715	<b>&lt; 2e-16</b>
mono-slow regular	-5.132	<b>3.44E-07</b>
mono-random	-5.405	<b>9.70E-08</b>
fast regular-slow regular	6.355	<b>4.18E-10</b>
fast regular-random	8.004	<b>3.33E-15</b>
slow regular-random	0.521	0.602

<sup>a</sup> The analysis was performed with R version 3.5.0. Post-hoc pairwise comparisons based on *z* statistics, following analysis of a general linear mixed model (GLMM), including multidrug  $\beta$ -lactam resistance as the response variable and sequence as fixed factors and replicate population as nested random factors. Representation of the model using R package *lme4*: *lmer(data=dfm12, res\_abc ~ evoexp\_ID + (1|line))*. All significant *P* values are shown in bold (*P* values were corrected for multiple testing using *false discovery rate*). Extinct lineages excluded.

**Figure 2-Supplementary Table 3. Statistical analysis of main evolution treatments for the multidrug  $\beta$ -lactam resistance after transfer 48 in Fig 2C<sup>a</sup>.**

Comparison	<i>z</i>	<i>P</i>
<b>Middle phase (transfer 48)</b>		
mono-fast regular	-6.933	<b>2.46E-11</b>
mono-slow regular	-2.763	<b>0.00686</b>
mono-random	-2.77	<b>0.00686</b>
fast regular-slow regular	4.034	<b>0.00011</b>
fast regular-random	5.193	<b>6.22E-07</b>
slow regular-random	0.426	0.66996

<sup>a</sup> The analysis was performed with R version 3.5.0. Post-hoc pairwise comparisons based on *z* statistics, following analysis of a general linear mixed model (GLMM), including multidrug  $\beta$ -lactam resistance as the response variable and sequence as fixed factors and replicate population as nested random factors. Representation of the model using R package *lme4*: *lmer(data=dfm48, res\_abc ~ evoexp\_ID + (1|line))*. All significant *P* values are shown in bold (*P* values were corrected for multiple testing using *false discovery rate*). Extinct lineages excluded.

**Figure 2-Supplementary Table 4. Statistical analysis of main evolution treatments for Shannon diversity in Figs. 2B and 2C<sup>a</sup>.**

Variable	Mean squares	$F_{1,4}$	$P$
Main treatment type	0.0656	0.7388	0.57349
Transfer	0.5513	6.206	<b>0.01893</b>

<sup>a</sup> The analysis of variance for the response variable within-population Shannon diversity  $H$  was performed with R version 3.5.0. Significant probabilities are given in bold.

**Figure 4-Supplementary Table 1. Minimum inhibitory concentrations (MICs) as determined by agar dilution for the three  $\beta$ -lactams.**

Antibiotic	MIC (mg/L)
CAR	50
CEF	1
DOR	0.06

**Figure 4-Supplementary Table 2. Likelihood Ratio Test to assess variation in spontaneous resistance rates in pairwise comparisons for the three  $\beta$ -lactam antibiotics<sup>a</sup>**

Antibiotic 1	Antibiotic 2	$P$
CAR	CEF	<b>&lt;0.0001</b>
CAR	DOR	<b>&lt;0.0001</b>
CEF	DOR	<b>&lt;0.01</b>

<sup>a</sup> The analysis was performed with the package rSalvador in R version 3.6.3. Significant  $P$  values are given in bold.

**Figure 4-Supplementary Table 3. Posthoc comparisons for phenotype of cross-resistance on secondary antibiotic using the false discovery rate<sup>a</sup>**

Comparison	$P$	Adjusted $P$
DOR – CEF	<b>0.000218</b>	<b>0.000327</b>
DOR – CAR	<b>0.0000319</b>	<b>0.0000957</b>
CEF – CAR	0.364	0.364

<sup>a</sup> The analysis was performed with the package rcompanion in R version 3.6.3. Significant  $P$  values are given in bold.

**Figure 5-Supplementary Table 1. Analysis of variance of the consequences of the main treatment type on the three measured responses for season 12 of the triple  $\beta$ -lactam experiment<sup>a</sup>**

Response	Adjusted $R^2$	$F_{2,9}$	$P$
Extinction	0.63	10.19	<b>0.0049</b>
Biomass increase	-0.22	0.003	0.997
Multidrug resistance	0.28	3.09	0.0953

<sup>a</sup> The analysis was performed with R version 3.5.1. Separate analyses were performed for each response variable. The subscript for the  $F$  ratio indicates the degrees of freedom. The  $P$  value gives the probability of the difference between a minimal and the defined model that includes the main treatment types as fixed factors. Significant probabilities are given in bold. The distribution of the response data is consistent with normality (Shapiro-Wilk test,  $W>0.89$ ,  $P>0.15$ ) and homogeneity of variance (Bartlett's test,  $\kappa^2<0.88$ , d.f.=2,  $P>0.64$ ).

**Figure 5-Supplementary Table 2. Posthoc comparison of the effect of the main treatment types on extinction for the triple  $\beta$ -lactam experiment using the false-discovery rate<sup>a</sup>**

Comparison	$P$
Fast regular – slow regular	<b>0.0076</b>
Fast regular – random	>0.99
Slow regular – random	<b>0.0064</b>

<sup>a</sup> The analysis was performed with R version 3.5.1. The posthoc test was only performed for the response variable, for which the main treatment type had a significant effect (see above table). Significant probabilities are given in bold.

**Figure 5-Supplementary Table 3. General linear model analysis of the consequences of the experimental predictors switching rate and irregularity on the three measured responses for season 12 of the triple  $\beta$ -lactam experiment<sup>a</sup>**

Response	Adjusted $R^2$	$F_{2,9}$	$P$
Extinction	0.68	12.48	<b>0.0025</b>
Biomass increase	-0.21	0.03	0.9667
Multidrug resistance	0.32	3.60	0.0712

<sup>a</sup> The analysis was performed with R version 3.5.1. Separate analyses were performed for each response variable. The subscript for the  $F$  ratio indicates the degrees of freedom. The  $P$  value gives the probability of the difference between a minimal and the defined model that includes switching rate and irregularity as fixed factors. Significant probabilities are given in bold.

**Figure 5-Supplementary Table 4. Main effect tests for the consequences of switching rate and irregularity on extinction and multidrug resistance (MDR) for season 12 of the triple  $\beta$ -lactam evolution experiment<sup>a</sup>**

<b>Factor</b>	<b>Extinction</b>		<b>MDR</b>	
	<i>F</i> <sub>1</sub>	<i>P</i>	<i>F</i> <sub>1</sub>	<i>P</i>
Switching rate	14.44	<b>0.0042</b>	3.0	0.1172
Irregularity	10.53	<b>0.0101</b>	4.19	0.0711

<sup>a</sup> Effect tests were only performed for response variables, for which the GLM analysis revealed at least a statistical trend ( $P < 0.1$ ) of an improvement of the defined model over a minimal model, thus only including extinction and multidrug resistance (MDR). See above table for the results. The effect tests were performed with R version 3.5.1. The subscript of the *F* ratio denotes the degrees of freedom. Significant probabilities are given in bold.

**Figure 5-Supplementary Table 5. General linear model analysis of the consequences of the three considered biological predictors, cumulative probability of spontaneous resistance, cross-resistance, and hysteresis on the three measured responses for season 12 of the triple  $\beta$ -lactam experiment<sup>a</sup>**

<b>Response</b>	<b>Adjusted <i>R</i><sup>2</sup></b>	<b><i>F</i><sub>3,8</sub></b>	<b><i>P</i></b>
Extinction	0.52	4.91	<b>0.0320</b>
Biomass increase	-0.01	0.97	0.4516
Multidrug resistance	0.22	2.02	0.1899

<sup>a</sup> The analysis was performed with R version 3.5.1. Separate analyses were performed for each response variable. The *P* value gives the probability of the difference between a minimal and the defined model that includes cumulative probability of spontaneous resistance, cross-resistance, and cumulative hysteresis level as fixed factors. Significant probabilities are given in bold.



**Figure 5-Supplementary Table 6. Main effect tests for the consequences of the three considered biological predictors, cumulative probability of spontaneous resistance, cross-resistance, and hysteresis on extinction of the triple  $\beta$ -lactam evolution experiment<sup>a</sup>**

<b>Factor</b>	<b>Extinction</b>	
	<b><i>F</i><sub>1</sub></b>	<b><i>P</i></b>
Spontaneous resistance	4.14	0.0763
Collateral effects	10.42	<b>0.0121</b>
Hysteresis	0.16	0.7015

<sup>a</sup> Effect tests were only performed for the response variable, for which the GLM analysis revealed a significant improvement of the defined model over a minimal model, thus only including extinction. See above table for the results. The effect tests were performed with R version 3.5.1. The subscript of the F ratio denotes the degrees of freedom. Significant probabilities are given in bold.

### ROBUST COLLATERAL SENSITIVITY TO AMINOGLYCOSIDE ANTIBIOTICS DUE TO CIPROFLOXACIN RESISTANCE EVOLUTION IN *PSEUDOMONAS AERUGINOSA*

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Antibiotic resistance is an evolutionary problem at its core. Therefore, antibiotic treatments need to account for evolution to be able to counter resistance and sustainably treat infections. Collateral sensitivity based strategies are a promising example of such treatments. Collateral sensitivity is the evolution of hyper susceptibility to one antibiotic upon resistance evolution to another. The inclusion of this trade-off in sequential and combination treatments has been demonstrated to successfully constrain resistance evolution *in vitro*. The translation of collateral sensitivity for clinical use, however, has faced some problems. One of the challenges is that collateral sensitivity tradeoffs can vary with the exact resistance mutation and genomic backgrounds. Consequently, the repeated evolution of a given collateral sensitivity tradeoff across species, different strains in a species, or multiple bacteria in a population cannot be guaranteed. In this study, we tested the robustness of collateral sensitivity against four antibiotics observed upon ciprofloxacin resistance evolution in *Pseudomonas aeruginosa* strain PA14, across the genomic diversity found in the species and two different evolutionary trajectories commonly observed in treatments. We found that collateral sensitivity to the tested aminoglycoside antibiotics was conserved across genomic backgrounds representative of the species-wide diversity and different evolutionary trajectories such as long-term evolved populations and spontaneous mutants. Of the remaining antibiotics, collateral sensitivity was either not observed or inconsistently observed. Our results identify collateral sensitivity to the aminoglycosides due to ciprofloxacin resistance evolution as a conserved trade-off valuable for the treatment of *P. aeruginosa* dominated infections.

## Introduction

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Bacteria rapidly evolve resistance to antibiotics, in clinical and non-clinical settings, rendering them ineffective<sup>1–3</sup>. With a dwindling supply of new antibiotics, the extensive use and misuse of antibiotics have ushered in an antimicrobial resistance (AMR) crisis<sup>4</sup>. A recent study estimated approximately 4.95 million deaths worldwide associated with bacterial AMR in 2019<sup>5</sup>, which is nearly three-quarters of the cumulative deaths due to COVID-19 globally in three years of the pandemic<sup>6</sup>. Accordingly, the development of sustainable antibiotic treatment methods is now of paramount importance. Evolution-informed antibiotic treatments which target bacteria while simultaneously limiting resistance evolution offer such an approach<sup>7,8</sup>. Among these, collateral sensitivity (CS) based strategies have shown promise in recent years<sup>9</sup>.

CS refers to increased sensitivity to one antibiotic induced by the evolution of resistance to a different antibiotic. It is usually contrasted with cross-resistance (CR) which is the induced resistance to one antibiotic upon evolving resistance to another<sup>10,11</sup>. Originally described in 1952<sup>10</sup>, the field of antibiotic CS has seen major developments in the last decade. Extensive collateral networks have been mapped out using laboratory strains<sup>12–18</sup> and clinical isolates<sup>16,19–21</sup>. These have shown that CS is prevalent across species but limited to a few antibiotic pairs. CS tradeoffs have further been shown to constrain resistance evolution when employed in sequential<sup>12,17,22–26</sup> and combination treatments<sup>27–30</sup> underscoring their potential as a sustainable antibiotic therapy. Lastly, some aspects of the underlying mechanisms have been uncovered, including changes in membrane potential<sup>13</sup>, increased activation of prodrug<sup>31</sup>, reduced degradation of the antibiotic<sup>32</sup>, downregulating recycling of antibiotic target<sup>31,32</sup>, and altered antibiotic binding sites<sup>33</sup>.

Despite its potential, the translation of CS to clinical use has met with some challenges. For it to be useful in the clinic, CS needs to be repeatable, however, the phenotype of CS appears to depend not only on the exact mechanism of resistance evolved<sup>15</sup> but also on the specific underlying mutation<sup>34</sup>. Consequently, CS has been observed to vary between experimental replicates performed on the same species with varying resistance mechanisms, arising due to the stochasticity of evolution<sup>15,35</sup>. Epistatic interactions between the resistance mechanism and genomic background have also been shown to affect CS, changing its expression across species<sup>36</sup>. Population size was also observed to affect CS<sup>37</sup>, with seemingly robust CS patterns in a population vanishing in larger populations as rare cross-resistance mutations became available. CS has also been shown to change over time during evolution<sup>38</sup>, likely due to changing mutational dynamics. CS was observed to also change with the bacterial lifestyle during evolution<sup>39</sup>, with selection strength<sup>40</sup>, and with the environment of assaying<sup>41,42</sup>. Where determined, these changes were attributable to different underlying genetic mutations. Finally, some CS tradeoffs were found to be unstable over time limiting their use<sup>43</sup>. These studies highlight the difficulties which can impede the use of CS in the clinic. There is an urgent need to identify robust CS tradeoffs to inform antibiotic treatments.

Progress towards the identification of robust CS tradeoffs has been made in recent years. Theoretical studies have developed methods to quantify the degree of CS in antibiotic pairs given the stochasticity of evolution<sup>35,44</sup>. The rationale is to identify pairs with the highest

probability for CS to be used in treatment. These studies have been complemented with experimental studies aiming to identify robust CS patterns across multiple replicates of a single strain<sup>45</sup>, across different AMR mutations in a single strain<sup>46,47</sup>, and clinical isolates<sup>16,19</sup>. These have been instrumental in identifying tradeoffs that show potential for repeatability across replicates and clinical mutations. They, however, do not consider substantial genomic variation, known to impact CS<sup>36</sup>. Indeed, a study aiming to identify conserved collateral effects across species could not find universal CS tradeoffs<sup>18</sup>. For a truly robust tradeoff, the effects of clinical and genomic variation on a CS tradeoff need to be considered.

In addition to the situations mentioned above, the CS phenotype of a population can also change with different evolutionary trajectories<sup>48</sup>. In a previous publication, we observed that the collateral effects of spontaneous mutants obtained from a fluctuation assay were different from those of long-term evolved populations. We hypothesize that this difference arises due to different underlying genetic changes. The spontaneous mutants likely have one resistance mutation while the long-term evolved populations accumulated multiple mutations over time. Both kinds of evolutionary trajectories can be expected to occur in a patient. A robust tradeoff should therefore be consistent across such different trajectories. Further, it was the collateral effects of the spontaneous mutants that were able to predict the efficacy of fast sequential therapy<sup>48</sup>. Fast sequential therapy ideally forces the bacteria to evolve *de novo* mutations at every antibiotic switch, the mutants at each switch are therefore spontaneous mutants. CS tradeoffs that constrain resistance evolution in fast sequential treatments should then be those of spontaneous mutants. This raises the question - should evolutionary trajectory-dependent collateral profiles be matched to the choice of sequential treatment?

In this study, we set out to test the robustness of a CS tradeoff between ciprofloxacin resistance and  $\beta$ -lactam and aminoglycoside sensitivity identified in an earlier study from our lab<sup>15</sup>. We assess robustness at two levels, genomic variation, and trajectory of evolution. We do so by studying a strain panel of *Pseudomonas aeruginosa*, representing the species-wide genetic diversity<sup>54</sup>, both as spontaneous resistance mutants and populations, which have been adapted to ciprofloxacin over 14 days.

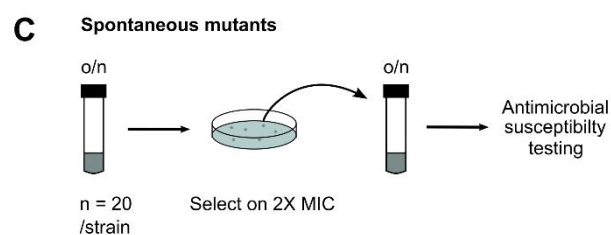
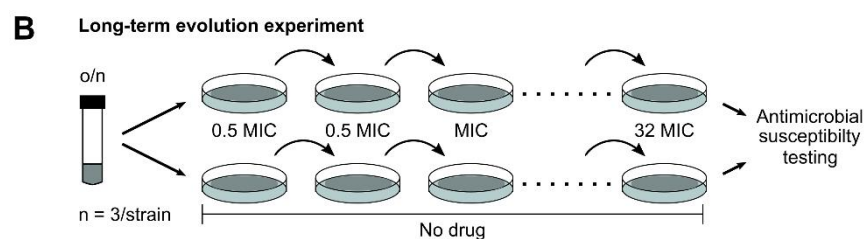
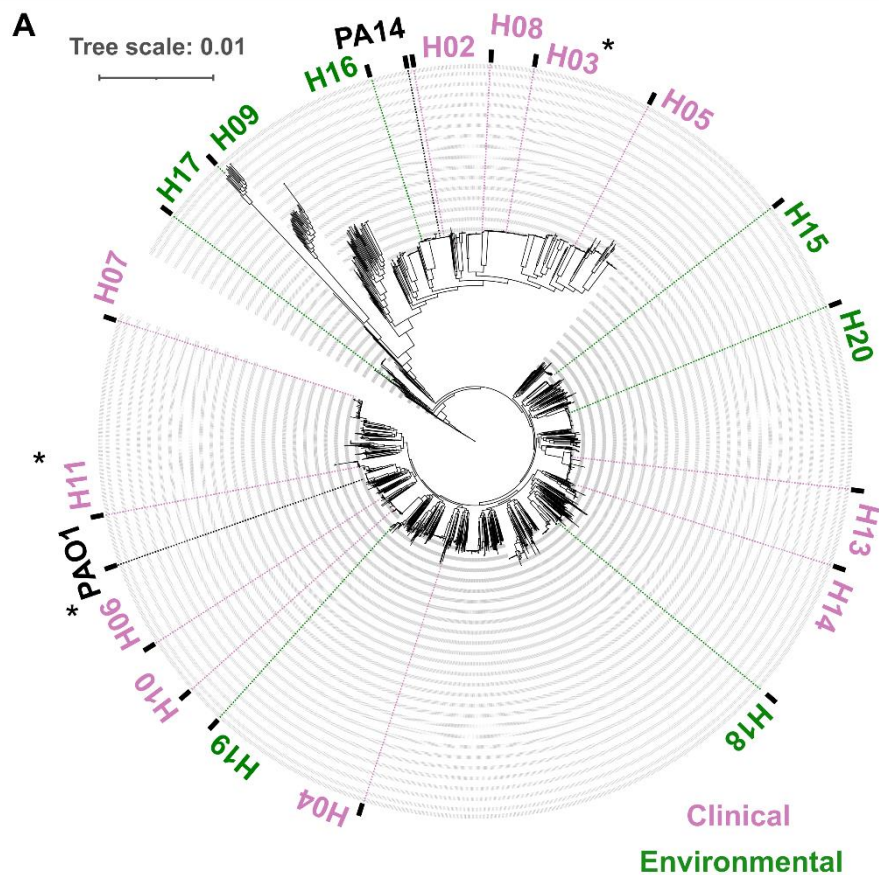
## Results

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### Selection for resistant bacteria

For the selection experiments, we used 16 strains from a strain panel of *P. aeruginosa*<sup>49,50</sup> along with the laboratory strains PAO1 and PA14<sup>51,52</sup>. The panel consists of clinical and environmental isolates which together span the observed species diversity in the taxa<sup>50</sup>(Fig. 1A). We carried out two separate selection experiments. In the first experiment, 20 biological replicates per strain (360 replicates total) were grown overnight in drug-free minimal medium and then plated on ciprofloxacin agar plates (2X minimal inhibitory concentration or MIC) to select resistant mutants (Fig. 1C). This approach is similar to the Luria-Delbrück fluctuation assay<sup>53</sup>. Resistant bacteria obtained this way were called spontaneous mutants. In the second selection experiment, 3 biological replicates per strain were grown on ciprofloxacin agar plates for a period of 15 days. Bacteria were passaged onto a new agar plate every day and the concentration of ciprofloxacin was doubled every third day. Appropriate no-drug controls were

included (resulting in a total of 108 replicates, Fig. 1B). Resistant populations that evolved this way were termed the long-term evolved populations. All ancestral (Table S1) and evolved bacteria were subjected to antimicrobial susceptibility testing to determine the minimum inhibitory concentration (MIC) on 5 antibiotics – ciprofloxacin (CIP), meropenem (MER), piperacillin/tazobactam (PTZ), gentamicin (GEN), and tobramycin (TOB) using ETEST®<sup>54</sup>.

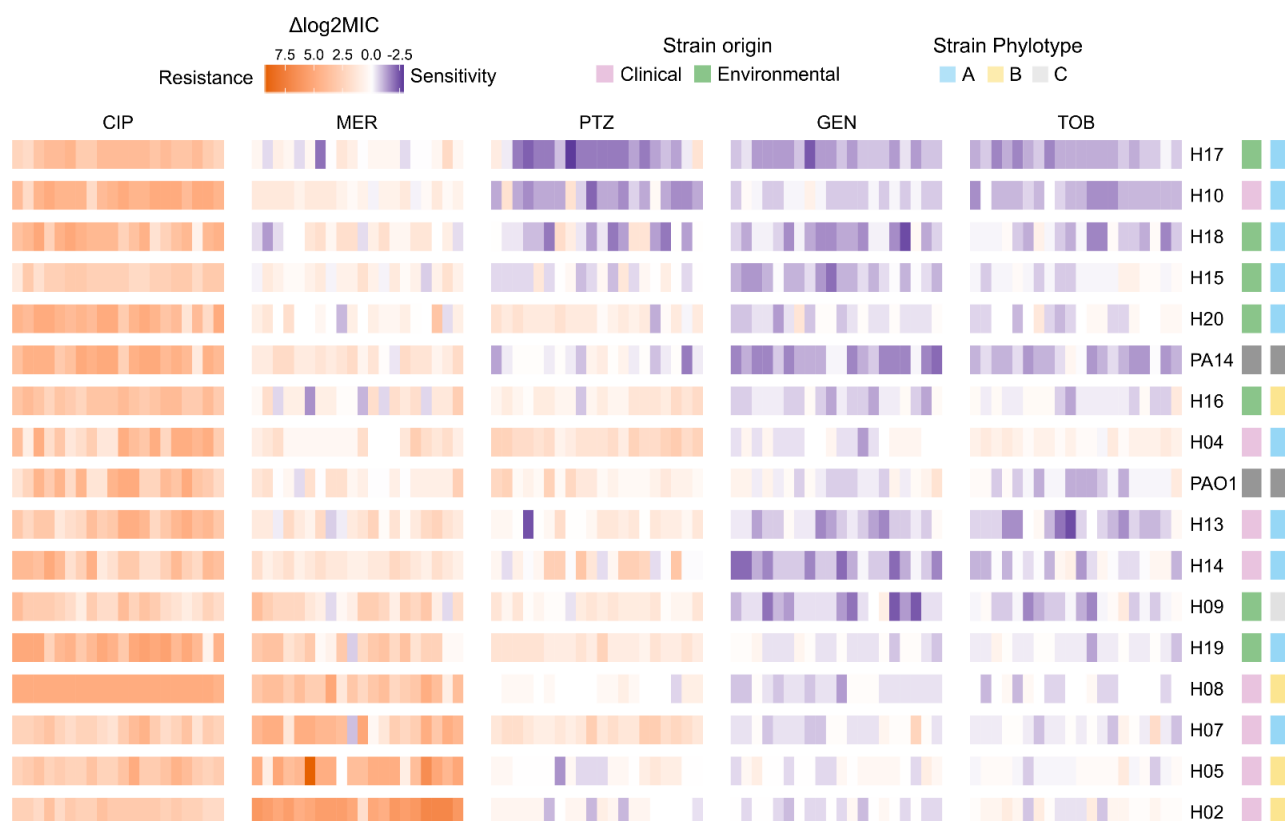


## Figure 1: Setup of the selection experiments

(A) The selection experiments were carried out using a strain panel of *Pseudomonas aeruginosa*. The strains in the panel spread out evenly on a phylogeny of published *P. aeruginosa* genomes<sup>53</sup> and are therefore a good representation of the genetic diversity in the species. Strains isolated from the clinic are in pink while those isolated from the environment are in green. Strains marked with a (\*) were not used for the experiments as they were resistant beyond the detection limit of the ETEST®. (B) Schematic of the long-term selection experiment. (continued on the next page) 3 replicates per strain were evolved under increasing concentrations of ciprofloxacin on agar plates. The experiment started at half the minimum inhibitory concentration (MIC) and the concentration doubled every third day. Appropriate no-drug controls were included. At the end of the experiment, the evolved populations were subjected to antimicrobial susceptibility testing to ciprofloxacin (CIP), meropenem (MER), piperacillin/tazobactam (PTZ), gentamicin (GEN), and tobramycin (TOB). (C) Schematic for the selection of spontaneous mutants. Overnight cultures of bacteria were grown in minimal medium without antibiotics and plated onto 2X MIC plates to select resistant mutants. 20 mutants/strain were generated using independent cultures. These were then used for antimicrobial susceptibility testing on the above-mentioned antibiotics.

## Antimicrobial susceptibility profiles of spontaneous mutants

We calculated the MIC fold-change of the evolved spontaneous mutants relative to the ancestor (Fig. 2). Fold-change on CIP represents the resistance of mutants to CIP while fold-change on the other antibiotics represents acquired collateral effects. We found that both, the antibiotic and the strain had an effect on the MIC fold-change (randomized ANOVA,  $F = 815.22$  and  $19.74$ , respectively,  $p < 0.0001$  for both). Resistance to CIP increased across all strains (Wilcoxon test,  $V = 58653$ ,  $p = 1$ ). Although some cases of CS were observed for MER and PTZ (12% and 28%, respectively), these were not consistent across replicates or strains (Wilcoxon test,  $V = 51512$  and  $39533$  for MER and PTZ,  $p = 1$  for both antibiotics). The observed CS on these antibiotics appeared to be driven by the genomic background of the strain as the strains showing CS did so on 3 out of the 4 antibiotics tested. CS on the aminoglycosides was pervasive with 76% and 65% of replicates showing CS on GEN and TOB, respectively. These were the only antibiotics to which sensitivity evolved reliably across strains (Wilcoxon test,  $V = 8102.5$  and  $14794$  for GEN and TOB,  $p < 0.0001$  for both). It is to be noted that 6% of all replicates did not differ from their ancestor in their antimicrobial susceptibility (AST). We assessed whether the strain origin (clinical vs environmental) or phylotype (A, B or C see ref<sup>50</sup>) could explain the observed CS pattern. The clustering of the CS phenotype, however, did not match that of the origin or phylotype ruling out this possibility (Fig. 2). We conclude that resistance to CIP in spontaneous mutants, results in robust CS to the aminoglycosides GEN and TOB across the species-wide genomic diversity in *P. aeruginosa*.



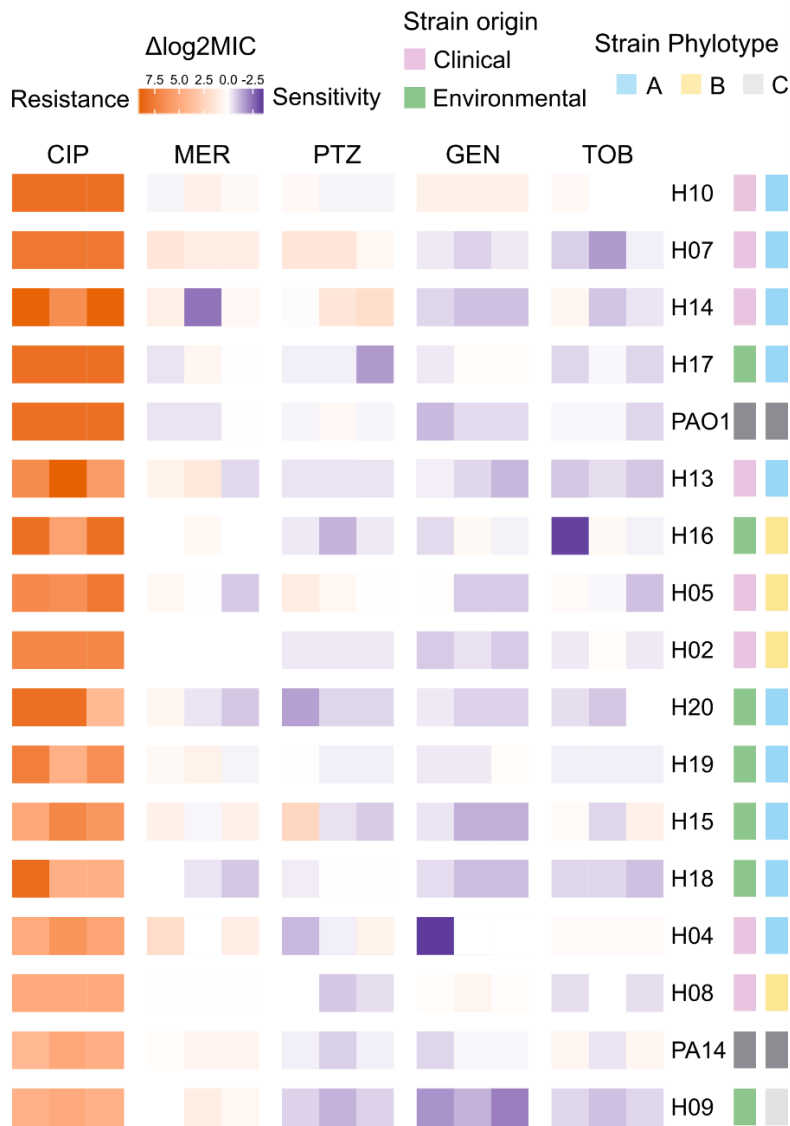
**Figure 2: Collateral effects to ciprofloxacin resistance in spontaneous mutants**

The heatmap shows the  $\log_2\text{MIC}$  fold-change of the evolved spontaneous mutants relative to their ancestors. AST was measured on five antibiotics indicated on the top. The strains are given on the right along with their origin and phylotype. Resistance and hypersensitivity are designated by orange and blue, respectively. AST on CIP denotes evolved resistance while AST on the other antibiotics denotes the evolved collateral effects. The strains are clustered using seriation. All mutants were resistant to CIP. CR dominated on MER and PTZ with only a fraction (12% and 28%, respectively) of replicates showing CS. Sensitivity to the aminoglycosides evolved more reliably with 76% and 65% of replicates showing CS on GEN and TOB respectively. Only 6% of all replicates showed no difference in collateral effects from their ancestor. The observed collateral pattern did not cluster by origin or phylogeny of the strains eliminating them as possible predictors of collateral effects. A total of 20 independent mutants were tested per strain. The strain H01 was tested (See Supplementary Fig. S1) but excluded from analysis as its MIC on GEN and TOB was beyond the detection limit of the ETEST®.



### **Antimicrobial susceptibility profiles of long-term evolved populations**

We calculated the MIC fold-change of the long-term populations that evolved in the presence of CIP relative to the ancestor (Fig. 3) and relative to the no-drug controls (Fig. S3) to account for any changes in susceptibility due to the experimental conditions. We found that both profiles were similar. We then carried out individual randomized ANOVA per antibiotic, with selection treatment and strain as factors. We found a significant effect of selection treatment and strain for all antibiotics (randomized ANOVA,  $p < 0.0001$  for all). As with the spontaneous mutants, resistance to CIP increased in all populations exposed to the antibiotic (Wilcoxon test,  $V = 0$ ,  $p < 0.0001$ ). CR was the dominant trend on MER (Wilcoxon test,  $V = 2301$ ,  $p < 0.01$ ) with only 38% of replicates showing CS on it. In contrast, CS towards PTZ dominated this time (Wilcoxon test,  $V = 5796$ ,  $p < 0.001$ ) with 72% replicates showing the phenotype. CS to the aminoglycosides evolved reliably across strains once again (Wilcoxon tests,  $V = 7239.5$  and  $6320.5$  for GEN and TOB,  $p < 0.0001$  for both), with 75% and 66% replicates showing CS on GEN and TOB, respectively. 8% of the replicates did not show any collateral effects. We moreover assessed if the strain origin or phylotype could predict the observed CS pattern (Fig. 3), however, this turned out not to be the case. We conclude that CS to the aminoglycosides GEN and TOB is robust not only across the species-wide genomic diversity in *P. aeruginosa* but also over the evaluated evolutionary trajectories. Furthermore, CS is more prevalent in the long-term populations as in addition to GEN and TOB it is also observed on PTZ.

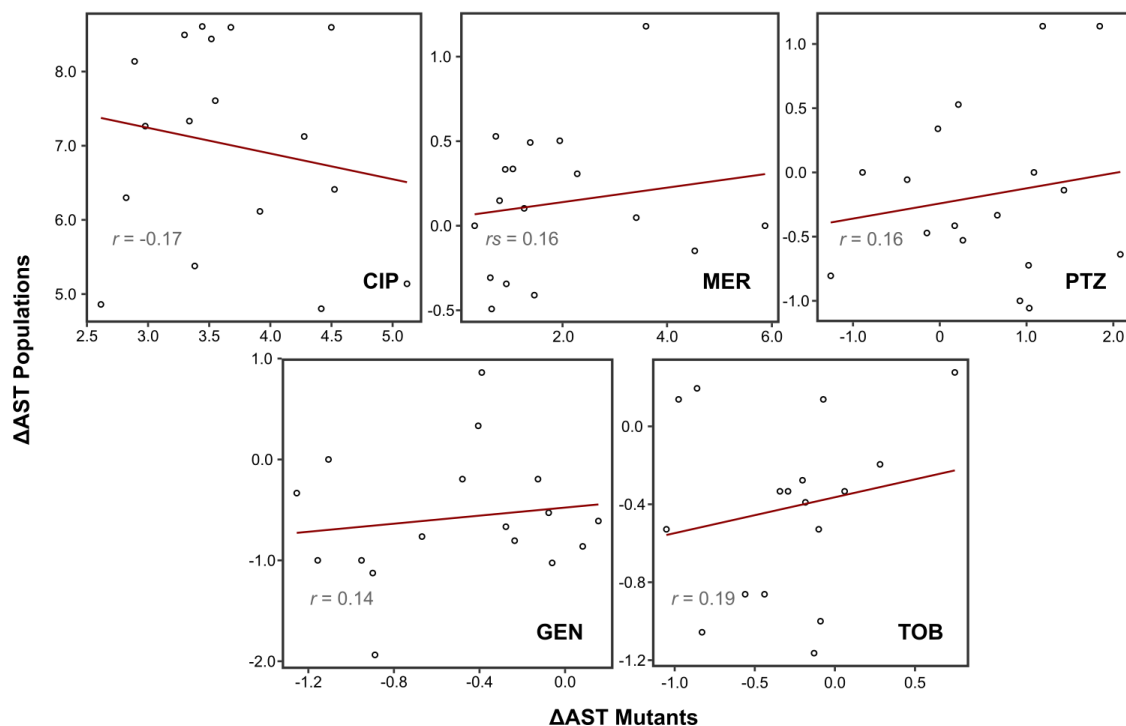


**Figure 3: Collateral effects to ciprofloxacin resistance in long-term evolved populations**

The  $\log_2\text{MIC}$  fold-change of the long-term evolved populations relative to the ancestor is plotted. Antibiotics on which AST was measured are given at the top. The strains, their origin, and their phylotype are given on the right. Resistance and hypersensitivity are depicted in orange and blue, respectively. AST on CIP is the evolved resistance while that on other antibiotics is evolved collateral effects. The strains are clustered using seriation. All populations that evolved in the presence of CIP were resistant to it. Collateral effects on MER tended towards CR with only 38% of replicates showing CS on it. Long-term evolved populations showed more CS on PTZ than spontaneous mutants with 72% of replicates showing the phenotype. CS on the aminoglycosides was robust with 75% and 66% replicates showing CS on GEN and TOB, respectively. A total of 8% of the replicates did not evolve any collateral effects. The origin and the phylotype of the strains were unable to predict their collateral effects. A total of 3 independent replicates were tested per strain. The strain H01 was tested (See Supplementary Fig. S3) but excluded from analysis as its MIC on GEN and TOB was beyond the detection limit of the ETEST®.

## **Relationship between the antimicrobial susceptibility profiles of the two evolutionary trajectories**

Having characterized the magnitude of and collateral effects due to CIP resistance in both the spontaneous mutants and the long-term evolved populations, we next wanted to test whether the AST profiles across the two trajectories were correlated. We tested this for the directionality of the AST as well as for the effect size. To test if the direction of AST (resistance or sensitivity) varied between the two evolutionary trajectories we performed logistic regressions and looked for significant changes in the slope. A change in direction of AST across the two trajectories for an antibiotic should result in a significant shift in the slope of the regression line. We found that this was indeed the case for PTZ (Logistic regression, slope = 0.039,  $p < 0.01$ ). In all other cases, the direction of AST remained unchanged. We then tested if the effect sizes of the AST were correlated. Scatter plots indicated that there appeared to be no such correlation (Fig. 4), a result which was verified by an ANCOVA (ANCOVA,  $F = 0.081$ ,  $p = 0.77$ ). We conclude that the direction of the AST between the two evolutionary trajectories remains largely unchanged except for PTZ, however, the effect sizes are not correlated. Predictions about the AST of one based on the AST of the other would be possible contingent on the antibiotic in question. These predictions nevertheless can only be made for the direction of the AST and not for the magnitude. We were further interested in whether the magnitude of resistance to CIP could be a predictor of the magnitude of the collateral effects evolved. We tested this individually in spontaneous mutants and in long-term evolved populations but did not find a significant relationship (ANCOVA,  $p > 0.05$  for all antibiotics).



**Figure 4: Correlation between the antimicrobial susceptibilities of the two evolutionary trajectories**

The change in AST for both the spontaneous mutants and the long-term populations was calculated relative to the ancestor for all 5 antibiotics and plotted. The X-axis shows the change in AST for the mutants while the Y-axis shows the change in AST for the long-term populations. Each data point is one strain. The red line depicts linear regression,  $r$  the Pearson's correlation coefficient, and  $r_s$  the Spearman's correlation coefficient. No significant correlation was observed between the ASTs of the two evolutionary trajectories.

## Discussion

CS informed antibiotic treatments are promising as sustainable treatments due to their ability to constrain resistance evolution<sup>12,17,27,29</sup>. Their translation into the clinic, however, has been subject to some difficulties. One of the challenges is that CS tradeoffs can vary with changing resistance mutations<sup>15,34</sup> and genomic background<sup>36</sup>. As a consequence, the repeated evolution of a given CS tradeoff across species, different strains in a species or multiple bacteria in a strain cannot be guaranteed. The same is true for bacteria which evolved resistance through different evolutionary trajectories. Repeatable, robust CS is a prerequisite for successful treatment. In this study, we observe robust CS to the aminoglycosides GEN and TOB upon resistance evolution to CIP in the opportunistic pathogen *Pseudomonas aeruginosa*. The evolved tradeoff is not only stable across the genomic variation found in the species but also across different evolutionary trajectories taken to resistance.

We decided to focus on this tradeoff because previous work from the lab indicated that resistance to CIP results in conserved CS to some  $\beta$ -lactams and aminoglycosides<sup>15</sup>. All 10 populations of *P. aeruginosa* strain PA14 resistant to CIP showed CS to the aminoglycosides

GEN and streptomycin and the  $\beta$ -lactam Carbenicillin. CS to PTZ and another  $\beta$ -lactam, Imipenem was observed in most but not all replicates<sup>15</sup>. Of all available candidates for a robust CS tradeoff, CIP resistance concomitant with aminoglycoside sensitivity has shown the most potential. It was observed to be conserved in clinical isolates of *Escherichia coli*<sup>19</sup> and *P. aeruginosa*<sup>16,47,55</sup>. Although these isolates belong to different genomic backgrounds, it is unclear how representative they are of species-wide diversity. Two recent studies profiled CS resulting from CIP resistance across different mutations of the same alleles in *Streptococcus pneumoniae*<sup>34</sup> and across different clinically relevant mutations in the strain PA14 of *P. aeruginosa*<sup>47</sup>. They found that resistance to CIP consistently gave CS to GEN and TOB respectively, across all tested mutations. Taken together, these studies show the reliability of CS due to CIP resistance across clinical variation, and some genomic variation. Genomic variation can have important consequences for the robustness of collateral sensitivity. A study that assessed collateral effects across the ESKAPE pathogens could not find conserved CS across species<sup>18</sup>. In that study, resistance to CIP resulted in CS to GEN in only 3 out of the 6 species and was not absolute within a species either. Interestingly, the same study found that CIP resistant *P. aeruginosa* did not show CS to GEN (3 of the 4 replicates tested), underscoring the need for significant replication in assessing the conservation of CS tradeoffs. We here tested CS due to CIP resistance in 18 different strains (with a total of 23 replicates per strain) of *P. aeruginosa* which represent the genomic diversity in the species. Even with the variation in genomic background, CS to the aminoglycosides GEN and TOB was found to be conserved. Our results, along with those mentioned above, demonstrate the utility of this tradeoff in the treatment of *P. aeruginosa* infections.

We tested the robustness of CS to ciprofloxacin resistance not only across genomic variation but also across different evolutionary trajectories taken to resistance. We found that the direction of collateral effects remained the same on all antibiotics except PTZ. The difference in selection conditions of the two trajectories are known to impact collateral effects. The spontaneous mutants grew planktonically before selection on agar while the long-term populations were continuously propagated on agar for a much longer time. Resistance mutations were acquired during the planktonic phase in the absence of antibiotics for the spontaneous mutants. For the long-term evolved populations, resistance mutations evolved during their growth on agar under increasing antibiotic selection. Not only is the strength of the selection known to impact collateral effects<sup>40</sup>, but also mode of growth<sup>39</sup>. Strength of selection can affect the available mutational spectrum and therefore likely collateral effects<sup>40</sup>. Growth on agar reduces access to antibiotics, promotes the development of niches, and thereby population structure. This ultimately can result in different targets of selection than planktonic populations and as a consequence different collateral effects than those of planktonic populations<sup>39</sup>. A recent study in *Enterococcus faecalis* showed that the time period of evolution can also influence collateral effects. Populations that evolve longer tend to have different collateral profiles than populations that evolved for a shorter period of time<sup>38</sup>. This is likely because of the accumulation of more mutations over time. The spontaneous mutants, therefore, can differ from long-term evolved populations in both quantity and type of mutations as they differ in all three selection conditions. This can in turn affect the evolved collateral effects. We have observed this in a previous publication<sup>48</sup>. These differences could explain why we saw a

change in the direction of collateral effects on PTZ or a lack of correlation in effect sizes of AST on other antibiotics. They also explain why we see a difference from the previous study from the lab, as there selection was in liquid media under increasing CIP concentrations<sup>15</sup>. It is important to note that, despite this, the CS to the aminoglycosides was observed to be conserved. This highlights that the robustness of this tradeoff extends beyond genomic variation. Further, collateral effects of spontaneous mutants have been proposed to better predict the success of sequential therapies<sup>48</sup>. Comparisons like the one we have made could help better tailor collateral effects to intended therapy. This would help to improve antibiotic treatments.

Why does CIP resistance result in conserved CS to GEN and TOB in *P. aeruginosa*? Resistance to CIP in this species has previously been shown to result from a broad range of different mutations: in DNA Gyrase, Topoisomerase IV, the efflux machinery, outer membrane diffusion channels, in genes such as *mutS*, *mutL*, and *nuoD*, and plasmid-based inactivating enzymes<sup>56–58</sup>. Some of these mutations, such as those causing upregulation of the MexXY-OprM efflux pump and the inactivating enzyme *aac(6′)-Ib-cr* also cause CR to aminoglycosides<sup>59,60</sup>. Why then are these not observed more often? The mutability of genes can differ and depends on many factors such as copy number, cost of mutation, length of the gene, sequences surrounding relevant bases for the resistance phenotype et cetera<sup>61</sup>. Indeed, most studies characterizing collateral effects to CIP have usually found mutations in DNA Gyrase, Topoisomerase IV, and specific efflux pumps/their regulators in *P. aeruginosa*<sup>16,18,47,55</sup>. This is also true in other species<sup>18,19,39</sup>. It could be that this bias in routes to resistance translates into a bias towards CS to GEN and TOB. This is likely not the entire picture, however, as three studies have reported additional routes to CIP resistance. This includes but is not limited to the porin OprD<sup>15</sup>, penicillin-binding proteins FtsI and FtsZ<sup>62</sup>, and the genes *orfH*, *orfJ*, and *orfN*<sup>24</sup>. These studies still found conserved CS to aminoglycosides. Perhaps then there is a paucity of CR mutations to the aminoglycosides in the CIP resistome of *P. aeruginosa*, making this phenotype rare. Overall, these studies suggest that conserved CS to GEN and TOB on CIP resistance in *P. aeruginosa* is likely multifactorial. Genomic analysis of the CIP resistant bacteria generated by this study would help shed light on this.

Our findings of a robust CS tradeoff between CIP resistance and aminoglycoside sensitivity could help guide the design of sustainable combination or sequential treatments for multi-lineage *P. aeruginosa* infections, such as those found in the cystic fibrosis lung<sup>63</sup>. To be able to do so, the ability of this tradeoff to constrain resistance evolution must first be evaluated, both in the lab and patient related conditions. We have characterized the collateral effects of two different evolutionary trajectories. Some collateral effects were found to be conserved while others were not. The matching of the differing collateral effects with the appropriate sequential treatments still needs to be tested. This would help improve the design of sequential treatment protocols and consequently their likelihood of success. Finally, the mechanisms responsible for conserved CS to the aminoglycosides are yet unknown. Genomic analysis to identify such mechanisms would be of value for precision medicine-based approaches.

## Materials and Methods

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### Materials

Experiments were conducted with a strain panel of *Pseudomonas aeruginosa* which represents genetic diversity observed in the species<sup>49,50</sup>. The lab strains PA14<sup>51</sup> and PAO1<sup>52</sup> were also included. Bacteria were grown in M9 minimal medium or agar (1.5%) supplemented with glucose (2 g/L), citrate (0.58 g/L), and casamino acids (1 g/L). Cultures and plates were incubated at 37°C with shaking at 150 rpm for liquid media. Antibiotics used were the quinolone ciprofloxacin, the  $\beta$ -lactams meropenem and piperacillin/tazobactam and the aminoglycosides gentamicin and tobramycin.

### Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) of antibiotics was measured using the Epsilonometer Test or ETEST®<sup>54</sup>. Bacterial cultures were grown for 18h following which they were diluted to an OD<sub>600</sub> of 0.08 and spread onto a plate using a cotton swab. ETEST® strips (Liofilchem) were placed onto the plate and the plate incubated for 24h after which the MIC was read at the intersection of the zone of inhibition and the test strip.

### Selection of spontaneous mutants

For the generation of single step mutants, 20 independent parallel cultures per strain were seeded in antibiotic free minimal medium and grown for 18h. The OD<sub>600</sub> of the cultures was adjusted to 0.08 and 100  $\mu$ L of the diluted culture plated on 2X MIC ciprofloxacin plates. Plates were incubated for another 24h to select for mutants. A single mutant per plate was then chosen for further analysis.

### Selection of long-term evolved populations

To obtain long-term evolved populations resistant to ciprofloxacin 3 independent parallel cultures per strain were seeded in antibiotic-free medium and grown for 18h. They were then diluted to 0.08 OD<sub>600</sub> and 100  $\mu$ L was plated onto 0.5X MIC ciprofloxacin plates and plates without the antibiotic. Every 24h 20% of the bacteria were transferred to a fresh plate of the corresponding treatment. The concentration of ciprofloxacin was doubled every third day. The experiment lasted a total of 15 days. Evolved populations were frozen in 10 % Dimethylsulfoxide (DMSO) on the last day. For logistical reasons, the 18 strains were split into two separate evolution experiments. Two ancestral strains, H04 and H13 were included in both as experimental controls. Results for them were found to be consistent across the experiments (Kolmogorov-Smirnov test,  $p < 0.05$ , Fig. S2).

### Statistics

Statistical analysis was carried out in R<sup>64</sup> using packages *car* and *lawstat*. The effect of strain and antibiotic on antimicrobial susceptibility in both spontaneous and long-term evolved populations was assessed using a randomization ANOVA. The general model of the ANOVA used was *anova(lm(log<sub>2</sub>MIC ~ Treatment \* Strain, data = subdata))*. The interaction model was replaced by an additive model in cases where the interaction term was insignificant. 10000

permutations of the model were run generate a null distribution using the F ratios as test statistics. P value was calculated as the probability of obtaining F ratios greater than or equal to the observed F ratios. Post hoc testing was carried out using pairwise Wilcoxon rank sum tests. Direction of antimicrobial susceptibility was assessed using Wilcoxon signed rank tests. Regression between the qualitative direction of antimicrobial susceptibility between the two evolutionary trajectories was carried out using the logistic regression model *glm(class ~ group, family = "binomial", data = data)*, Relationships between the antimicrobial susceptibility magnitudes of the two evolutionary trajectories and between the magnitude of CIP resistance and collateral effects were evaluated using the ANCOVA model *Anova(aov(lm(`Response` ~ `Predictor` + Antibiotic, data = data)), type = "III")*. In cases where the ANCOVA was significant, logistic regressions between predictor and response variables per antibiotic were carried out to judge where the difference lay.

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### **Competing interests statement**

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The authors declare no competing interests.



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# ROBUST COLLATERAL SENSITIVITY TO AMINOGLYCOSIDE ANTIBIOTICS DUE TO CIPROFLOXACIN RESISTANCE EVOLUTION IN *PSEUDOMONAS AERUGINOSA* SUPPLEMENTARY INFORMATION

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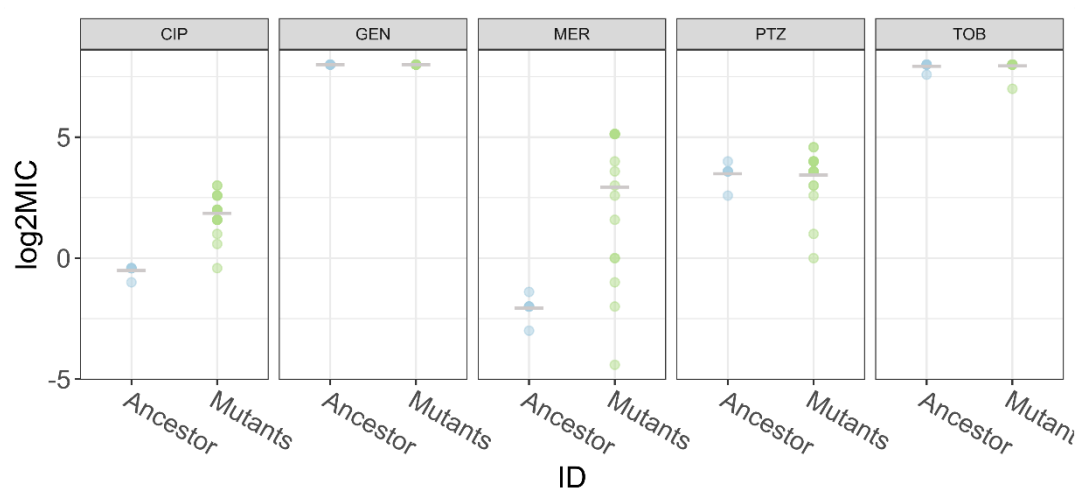
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**Supplementary Table S1: Minimum inhibitory concentrations (ug/mL) of the ancestral strains used in the experiment**

<b>Sr. No.</b>	<b>Strain</b>	<b>Replicate</b>	<b>CIP</b>	<b>GEN</b>	<b>TOB</b>	<b>MER</b>	<b>PTZ</b>
1	PAO1	1	0.094	1.5	1.5	0.032	3
2	PAO1	2	0.125	0.75	2	0.023	2
3	PAO1	3	0.047	1.5	1.5	0.047	2
4	PA14	1	0.094	0.75	1.5	0.016	2
5	PA14	2	0.125	1	1	0.023	1.5
6	PA14	3	0.094	0.75	0.75	0.023	2
7	H01	1	0.75	>256	>256	0.38	12
8	H01	2	0.75	>256	>256	0.125	12
9	H01	3	0.75	>256	>256	0.25	16
10	H01	4	0.5	>256	>256	0.25	6
11	H01	5	0.75	>256	>256	0.25	12
12	H01	6	0.75	>256	192	0.25	12
13	H02	1	0.19	1	1.5	0.047	8
14	H02	2	0.19	2	1	0.047	8
15	H02	3	0.25	1.5	1.5	0.047	8
16	H03	1	32	64	64	0.094	12
17	H03	2	32	32	48	0.125	12
18	H03	3	32	64	64	0.19	12
19	H04	1	0.032	1	0.75	0.023	1.5
20	H04	2	0.047	1	1	0.023	2
21	H04	3	0.064	1	0.75	0.023	2
22	H05	1	0.125	2	1.5	0.064	8
23	H05	2	0.094	1	1.5	0.064	3
24	H05	3	0.125	1.5	2	0.023	8
25	H06	1	32	1.5	1.5	0.047	12
26	H06	2	32	1	1.5	0.047	16
27	H06	3	32	1.5	1.5	0.032	16
28	H07	1	0.094	1	2	0.023	3
29	H07	2	0.125	1.5	1.5	0.023	2
30	H07	3	0.125	1.5	2	0.023	4
31	H08	1	1	1	1.5	0.064	6
32	H08	2	0.75	1.5	1.5	0.125	6
33	H08	3	1	1.5	1.5	0.094	6
34	H09	1	0.125	2	1.5	0.023	2
35	H09	2	0.125	2	1.5	0.023	3
36	H09	3	0.094	2	2	0.023	3
37	H10	1	0.094	0.75	1.5	0.016	1.5
38	H10	2	0.094	0.75	1.5	0.016	2
39	H10	3	0.064	1	1.5	0.023	4
40	H11	1	32	1.5	2	0.094	8
41	H11	2	32	1.5	1.5	0.094	8
42	H11	3	32	1.5	1.5	0.094	8
43	H12	1	32	1.5	1	16	12

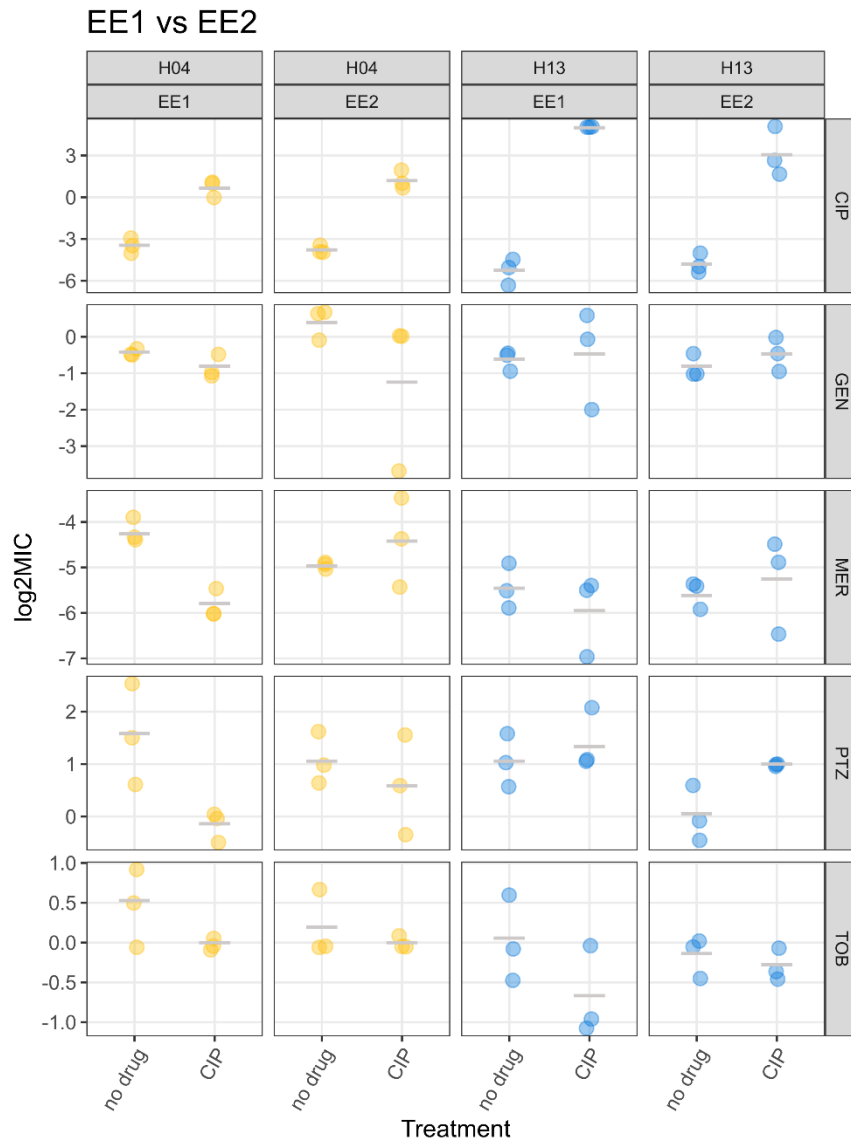
<b>44</b>	H12	2	32	1.5	1.5	12	8
<b>45</b>	H12	3	32	1.5	1.5	8	6
<b>46</b>	H13	1	0.047	1.5	1.5	0.016	2
<b>47</b>	H13	2	0.047	NA	NA	NA	NA
<b>48</b>	H13	3	0.047	1	1.5	0.023	4
<b>49</b>	H14	1	0.064	1.5	0.75	0.012	1
<b>50</b>	H14	2	0.064	2	1	0.012	0.75
<b>51</b>	H14	3	0.032	1.5	1.5	0.012	1.5
<b>52</b>	H15	1	0.032	1	0.75	0.032	0.5
<b>53</b>	H15	2	0.064	1.5	0.75	0.023	0.75
<b>54</b>	H15	3	0.064	0.75	1	0.023	1
<b>55</b>	H16	1	0.094	0.75	1.5	0.047	3
<b>56</b>	H16	2	0.125	1.5	0.75	0.047	3
<b>57</b>	H16	3	0.047	1.5	1.5	0.047	2
<b>58</b>	H17	1	0.064	1.5	2	0.032	4
<b>59</b>	H17	2	0.094	1.5	1.5	0.047	3
<b>60</b>	H17	3	0.094	1	1.5	0.023	4
<b>61</b>	H18	1	0.064	2	1.5	0.032	3
<b>62</b>	H18	2	0.064	3	1.5	0.032	3
<b>63</b>	H18	3	0.094	2	2	0.032	6
<b>64</b>	H19	1	0.094	1.5	2	0.047	4
<b>65</b>	H19	2	0.047	1.5	2	0.023	3
<b>66</b>	H19	3	0.094	1	1.5	0.047	4
<b>67</b>	H20	1	0.094	1.5	1.5	0.032	3
<b>68</b>	H20	2	0.064	1	1.5	0.032	3
<b>69</b>	H20	3	0.094	1.5	1.5	0.032	4



**Supplementary Figure S1: Antimicrobial susceptibility of H01 spontaneous mutants.**

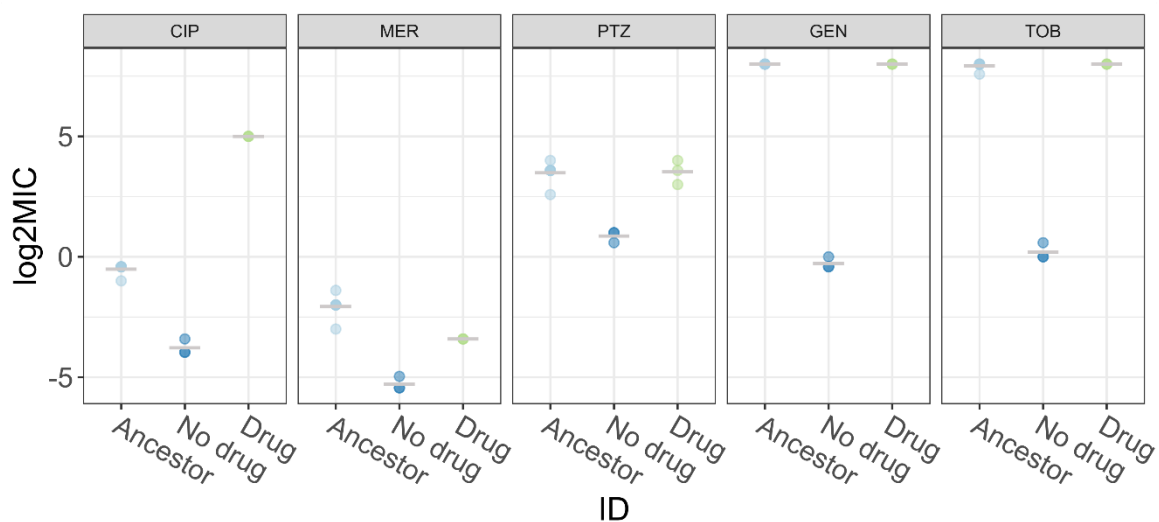
The figure shows the log<sub>2</sub>MIC of the spontaneous mutants derived from the strain H01 on the 5 antibiotics. Log<sub>2</sub>MIC is plotted on the Y axis while the X-axis shows the bacteria tested. Each panel corresponds to one antibiotic. The thick line on top of the data points represents the mean. MIC was determined for 3 replicates of the ancestor and 20 mutants.





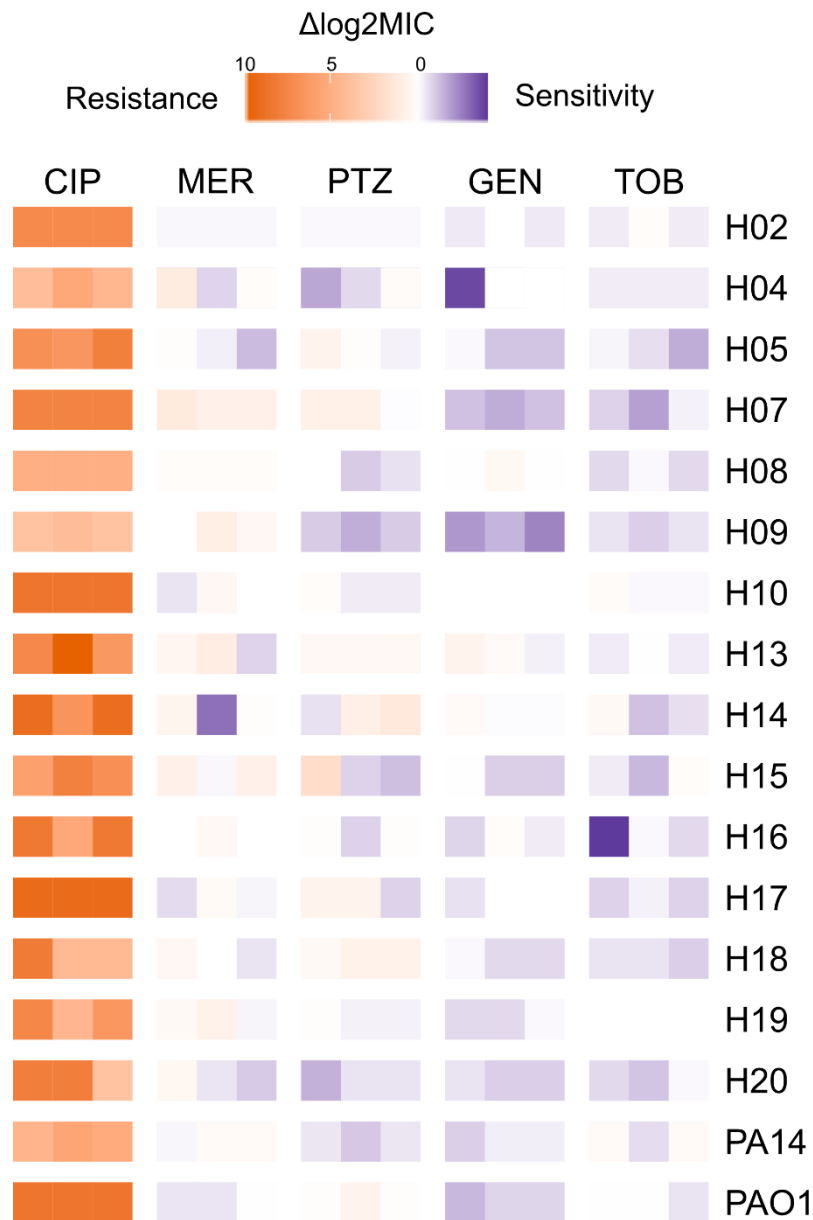
**Supplementary Figure S2: Comparison of evolved antimicrobial susceptibility of H04 and H13 between the two long-term selection experiments**

The figure shows the log<sub>2</sub>MIC of the long-term evolved populations of strains H04 and H13 in the two long-term selection experiments. Briefly, due to logistic reasons, the long-term selection experiment was split into two. Strains H04 and H13 were evolved in both to control for any difference between the two. The two treatments in the selection experiment are given on the X-axis. The log<sub>2</sub>MICs are plotted on the Y-axis. The strain and the selection experiment are mentioned at the top. Antibiotics on which MIC was tested are given on the right. Overall, we did not see any difference in the distribution of the MICs between the two evolution experiments (Kolmogorov-Smirnov test,  $p < 0.05$ )



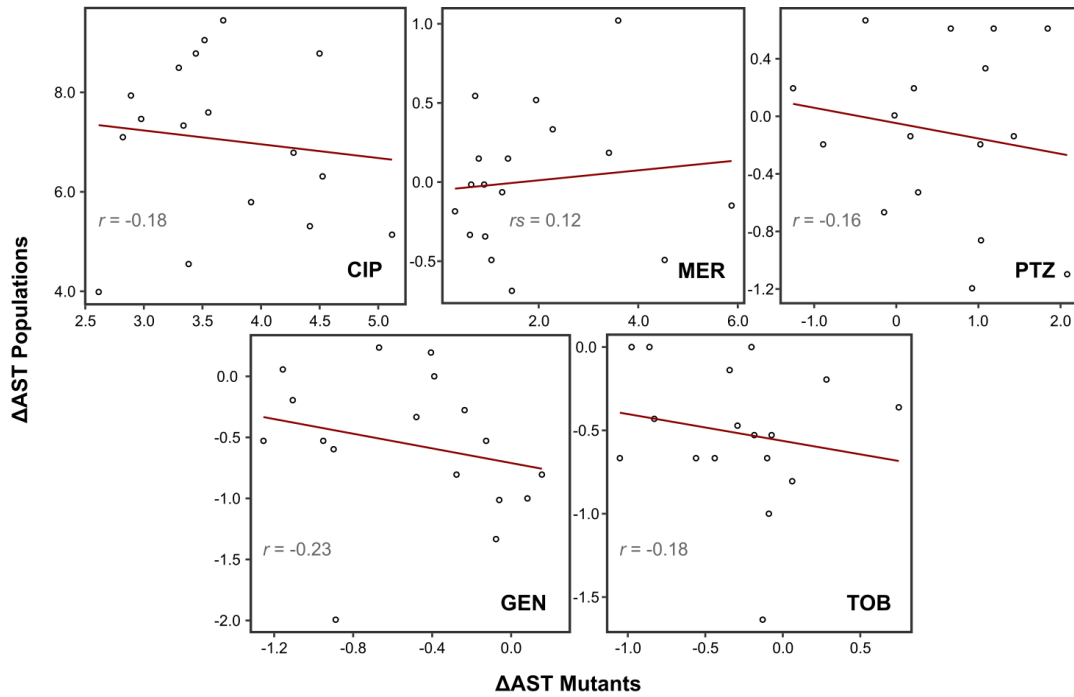
### Supplementary Figure S3: Antimicrobial susceptibility of H01 long-term populations

Log2MICs of the ancestral and long-term evolved populations of the strain H01 on the 5 antibiotics are shown. The identity of the population is given on the X-axis while the log2MICs are plotted on the Y-axis. Each panel corresponds to one antibiotic. The mean log2MIC is plotted as a thick line on top of the data points. H01 showed the greatest loss in resistance upon drug-free evolution amongst all strains tested. 3 replicates were tested for the ancestor, no-drug evolved and drug-evolved populations each.



**Supplementary Figure S4: Collateral effects to ciprofloxacin resistance in long-term evolved populations relative to the no-drug controls**

The  $\log_2\text{MIC}$  fold-change of the long-term evolved populations relative to the ancestor is plotted. Antibiotics on which AST was measured are given at the top. The strains, their origin, and their phylotype are given on the right. Resistance and hypersensitivity are depicted in orange and blue, respectively. AST on CIP is the evolved resistance while that on other antibiotics is evolved collateral effects. A total of 3 independent replicates were tested per strain per treatment. The strain H01 was tested (See Supplementary Fig. S3) but excluded from analysis as its MIC on GEN and TOB was beyond the detection limit of the Etest.



**Supplementary Figure S5: Correlation between the antimicrobial susceptibilities of the two evolutionary trajectories when normalized to the no-drug**

The change in AST for the spontaneous mutants relative to the ancestor and the long-term populations relative to the no-drug was calculated for all 5 antibiotics and plotted. The X-axis shows the change in AST for the mutants while the Y-axis shows the change in AST for the long-term populations. Each data point is one strain. The red line depicts linear regression,  $r$  the Pearson's correlation coefficient, and  $r_s$  the Spearman's correlation coefficient. No significant correlation was observed between the ASTs of the two evolutionary trajectories even with the change in the normalisation procedure.

**Table S2: Comparability of the two sets of long-term evolution experiments**

The long-term evolution experiment had to be split into two due to logistic constraints. To evaluate comparability, we included two strains, H04 and H13 in both. We performed a Kolmogorov-Smirnov test on the two strains to assess whether cumulative distributions of the log<sub>2</sub>(MIC) readings for these strains differed between the experimental runs.

	No drug control		Ciprofloxacin treated	
	<i>D statistic</i>	<i>p-value</i>	<i>D statistic</i>	<i>p-value</i>
<b>Strain</b>	<b>H04</b>			
CIP	0.3333333	1.0000	0.3333333	1.0000
GEN	1.0000000	0.1000	0.6666667	0.4000
TOB	0.3333333	1.0000	0.0000000	1.0000
MER	1.0000000	0.1000	0.6666667	0.6000
PTZ	0.3333333	1.0000	0.6666667	0.6000
<b>Strain</b>	<b>H13</b>			
CIP	0.3333333	1.0000	0.6666667	0.4000
GEN	0.3333333	1.0000	0.3333333	1.0000
TOB	0.3333333	1.0000	0.6666667	0.6000
MER	0.3333333	1.0000	0.6666667	0.6000
PTZ	0.6666667	0.6000	0.3333333	1.0000

**Table S3: Variation of fold-changes in MIC in the long-term evolution experiment:  
Statistics for testing premises of parametric ANOVA**

To test whether the average fold-changes in MIC varied significantly for different strains and treatment conditions, parametric full-factorial ANOVAs were executed for each of the 5 antibiotics. However, tests for normality of the residuals (Shapiro-Wilk tests) and for the residuals' homoscedasticity (Levene tests) have shown that parametric test assumptions are not fulfilled. Thus, the ANOVA design has been randomized (see table S4) to reliably estimate p-values.

		<b>CIP</b>	<b>GEN</b>	<b>TOB</b>	<b>MER</b>	<b>PTZ</b>
<b>Shapiro-Wilk test</b>	<i>Test statistic</i>	0.8971545	0.885242	0.9104469	0.9498311	0.9576868
	<i>W</i>					
	<i>p value</i>	<b>0.0000000</b>	<b>0.0000000</b>	<b>0.0000000</b>	<b>0.0000069</b>	<b>0.0000397</b>
<b>Levene test: Strain</b>	<i>Test statistic</i>	2.5209539	1.266299	1.8197737	1.7909143	2.0604709
	<i>p value</i>	<b>0.0007556</b>	0.2096712	<b>0.0227209</b>	<b>0.0258441</b>	<b>0.0075066</b>
<b>Levene test: Treatment</b>	<i>Test statistic</i>	12.929212	3.192204	6.8811164	7.4288432	3.7896780
	<i>p value</i>	<b>0.0000058</b>	<b>0.0435123</b>	<b>0.0013319</b>	<b>0.0008031</b>	<b>0.0245129</b>

**Table S4: Variation of fold-changes in MIC in the long-term evolution experiment: Randomized ANOVA**

To overcome the violation of parametric ANOVA requirements, full-factorial ANOVAs designs were randomized (in 100000 permutations) to test whether resistances to the 5 antibiotics used (CIP, GEN, TOB, MER, and PTZ) varied with the strains and treatment conditions in the long-term evolution experiment. Although test statistics, parameters, and p-values are shown for the interaction terms in the table below, the F statistics, parameters, and p-values have been only derived from additive models (models without interaction terms) in cases interactions turned out to be non-significant (gray entries).

		<b>CIP</b>	<b>GEN</b>	<b>TOB</b>	<b>MER</b>	<b>PTZ</b>
<b>Treatment</b>	<i>F statistic</i>	1470.0131	115.465	17.500	24.095	27.1622
	<i>df</i>	2	2	2	2	2
	<i>p-value</i>	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
<b>Strain</b>	<i>F statistic</i>	70.6081	91.677	19.392	30.243	21.8878
	<i>df</i>	21	21	21	21	21
	<i>p-value</i>	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
<b>Interaction (Strain:Interaction)</b>	<i>F statistic</i>	5.986186	21.63078	20.21638	2.735594	3.109377
	<i>df</i>	34	34	34	34	34
	<i>p-value</i>	< 0.0001	0.0034	0.3255	0.4815	< 0.0001

**Table S5: Variation of fold-changes in MIC in the long-term evolution experiment: Post-hoc tests**

To test for which antibiotics the MIC fold-changes differed significantly, Wilcoxon rank sum tests have been performed on the differences between all replicates from each treatment group combination. Only treatment group combinations of interest (ancestral vs. ciprofloxacin treated and no drug control vs. ciprofloxacin treated) have been tested.

Antibiotic	Calculation	Wilcoxon rank sum test		Median difference	Mean difference
		<i>Test statistic</i> <i>V</i>	<i>p-value</i>		
CIP	ancestral - ciprofloxacin treated	0.000000	<b>7.424257e-30</b>	-7.000000	-7.001063
CIP	no drug control - ciprofloxacin treated	0.000000	<b>9.467431e-36</b>	-7.395929	-6.984841
GEN	ancestral - ciprofloxacin treated	7239.5	<b>9.032696e-14</b>	0.5849625	0.6072078
GEN	no drug control - ciprofloxacin treated	10291.0	<b>2.534519e-12</b>	0.4150375	0.5121945
TOB	ancestral - ciprofloxacin treated	6320.5	<b>3.384688e-11</b>	0.4150375	0.3921463
TOB	no drug control - ciprofloxacin treated	8530.0	<b>2.453896e-18</b>	0.4150375	0.4842995
MER	ancestral - ciprofloxacin treated	2301	<b>0.003842156</b>	0.0000000	-0.14433096
MER	no drug control - ciprofloxacin treated	6506	0.593549552	0.0000000	0.06727671
PTZ	ancestral - ciprofloxacin treated	5796	<b>0.0006909467</b>	0.4150375	0.2057132
PTZ	no drug control - ciprofloxacin treated	7987	<b>0.0304364545</b>	0.0000000	0.1413816



**Table S6: Comparison of MIC fold changes across evolutionary trajectories**

The log2MIC fold-changes from the long-term selection experiment as well as for the spontaneous CIP-resistant mutants have been binary coded depending on whether resistance (positive) or susceptibility(negative) to the antibiotics tested increased. A change in the direction of resistance/susceptibility evolution between the two evolutionary trajectories (spontaneous mutants vs. long-term populations) was tested with logistic regression for each antibiotic.

<b>Antibiotic</b>	<b><i>Intercept</i></b>	<b><i>z</i> (<i>Intercept</i>)</b>	<b><i>p</i> (<i>Intercept</i>)</b>	<b><i>Slope</i></b>	<b><i>z (Slope)</i></b>	<b><i>p (Slope)</i></b>
<b>long-term populations relative to ancestral log2MIC values vs. spontaneous mutants relative to ancestral</b>						
<b>CIP</b>	25.57	0.000	1.0000	2.206e-06	0.000	1.0000
<b>GEN</b>	-1.540	-2.421	<b>0.0155</b>	-1.398e-15	0.000	1.0000
<b>MER</b>	1.1787	2.061	<b>0.0393</b>	18.3874	0.007	0.9944
<b>PTZ</b>	-1.1787	-2.061	<b>0.0393</b>	2.7191	3.179	<b>0.0015</b>
<b>TOB</b>	-1.1787	-2.061	<b>0.0393</b>	0.3032	0.388	0.6979
<b>long-term populations relative to control-evolved log2MIC values vs. spontaneous mutants relative to ancestral</b>						
<b>CIP</b>	25.57	0.000	1.0000	2.206e-06	0.000	1.0000
<b>GEN</b>	-1.1787	-2.061	<b>0.0393</b>	-0.3618	-0.423	0.6723
<b>MER</b>	0.6061	1.194	0.232	18.9599	0.007	0.994
<b>PTZ</b>	-0.1178	-0.242	0.8085	1.6582	2.072	<b>0.0383</b>
<b>TOB</b>	-1.5404	-2.421	<b>0.0155</b>	0.6650	0.802	0.4228

**Table S7: Correlations between antimicrobial susceptibilities between evolutionary trajectories**

We investigated whether there's a significant positive or negative correlation between the antimicrobial susceptibilities between both evolutionary trajectories (long-term evolution as well as spontaneous mutants) by carrying out correlation tests. In case the log2MIC fold-changes were normally distributed for every antibiotic-trajectory combination, a Pearson product moment-correlation (r) has been used (df = 15). Otherwise, the Spearman rank correlation coefficient (rs) has been tested.

Antibiotic	Correlation	Coefficient	Test statistic	p-value
<b>long-term populations relative to ancestral log2MIC values vs. spontaneous mutants relative to ancestral</b>				
<b>CIP</b>	r	-0.1742332	-0.6852842	0.5036181
<b>GEN</b>	r	0.1431704	0.5602686	0.5835678
<b>MER</b>	rs	0.2034314	650.0000000	0.4320626
<b>PTZ</b>	r	0.1639878	0.6438383	0.5294024
<b>TOB</b>	r	0.1888335	0.7447476	0.4679330
<b>long-term populations relative to control-evolved log2MIC values vs. spontaneous mutants relative to ancestral</b>				
<b>CIP</b>	r	-0.1174599	-0.4580915	0.6534530
<b>GEN</b>	r	-0.2370695	-0.9451088	0.3595796
<b>MER</b>	rs	0.1385653	702.9306961	0.5958538
<b>PTZ</b>	r	-0.1627728	-0.6389376	0.5324996
<b>TOB</b>	r	-0.1808814	-0.7122999	0.4872116

**Table S8: Independence of antimicrobial susceptibilities between evolutionary trajectories (randomized ANCOVA)**

Considering the non-normality and heteroscedasticity of fold-change numbers identified earlier (cf. table S3), a randomized ANCOVA has been performed to test whether the resistance or susceptibility in the long-term evolved populations act as predictors for the MIC fold-changes in the spontaneous mutants. However, the p-values indicate that this doesn't seem to be the case.

Source of variation	Sum of squares	<i>F statistic</i>	<i>df</i>	<i>p-value</i>
<b>long-term populations relative to ancestral log2MIC values vs. spontaneous mutants relative to ancestral</b>				
Intercept	11.217			
Long-term evolved	0.070	0.0811331	1	0.7744
Antibiotic	53.719	15.6319443	4	<b>&lt; 0.0001</b>
Residuals	67.871		79	
<b>long-term populations relative to control-evolved log2MIC values vs. spontaneous mutants relative to ancestral</b>				
Intercept	19.279			
Long-term evolved	0.253	0.2947505	1	0.5904
Antibiotic	62.426	18.2147300	4	<b>&lt; 0.0001</b>
Residuals	67.688		79	

**Table S9: Parametric ANCOVA: Magnitude of resistance to CIP is not a predictor for the magnitude of collateral effects**

Source of variation	Sum of squares	<i>F statistic</i>	<i>df</i>	<i>p-value</i>
<b>Resistance to antibiotic after long-term evolution, compared to ancestral readings</b>				
Resistance to CIP	0.213	0.693	1	0.40819
Antibiotics	4.915	5.332	3	<b>0.00245</b>
Residuals	19.359		63	
<b>Resistance to antibiotic after long-term evolution, compared to control-evolved readings</b>				
Resistance to CIP	1.354	5.491	1	<b>0.0223</b>
Antibiotics	4.146	5.603	3	<b>0.0018</b>
Residuals	15.538		63	
<b>Resistance to antibiotic after single step evolution, compared to ancestral readings</b>				
Resistance to CIP	4.600	0.788	1	0.378
Antibiotics	317.1	18.052	3	<b>&lt; 0.001</b>
Residuals	368.9		63	

**Table S10: Statistics for testing premises of parametric ANCOVA**

The Shapiro-Wilk tests (for normality of residuals) as well as the Breusch-Pagan tests (for homoscedasticity of residuals along the predictor variables) indicate that the requirements for the parametric ANCOVA are fulfilled and thus, that the estimated models' p-values should be reliable.

<b>Test</b>	<b>Test statistics</b>	<b><i>df</i></b>	<b><i>p value</i></b>
<b>Resistance to antibiotic after long-term evolution, compared to ancestral readings</b>			
Shapiro-Wilk test	0.97895		0.3045
Breusch-Pagan test	0.10826	1	0.7421
<b>Resistance to antibiotic after long-term evolution, compared to control-evolved readings</b>			
Shapiro-Wilk test	0.98555		0.6201
Breusch-Pagan test	2.2829	1	0.1308
<b>Resistance to antibiotic after single step evolution, compared to ancestral readings</b>			
Shapiro-Wilk test	0.97518		0.1924
Breusch-Pagan test	0.11467	1	0.7349

### EVOLUTION-INFORMED TREATMENTS CONSTRAIN ADAPTATION IN A GENETICALLY DIVERSE POPULATION OF *PSEUDOMONAS AERUGINOSA*

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Evolutionary processes are a prime cause of antibiotic resistance in bacteria. Evolution-informed treatments target these processes to select against resistance evolution. These treatments have mostly been evaluated with single strains of a species. Some sites of infection, however, can be colonized by multiple strains of a species. An example is the cystic fibrosis (CF) lung, which harbours many different genetic lineages of the pathogen *Pseudomonas aeruginosa*. Isolates of *P. aeruginosa* from the CF lung frequently interact with each other. Such interactions could accelerate resistance evolution through horizontal gene transfer or suppress growth and thereby evolution of resistance through competitive interactions. The impact of these interactions on the efficacy of evolution-informed treatments is not known. In this study, we set out to evaluate two evolution-informed treatments, sequential and combination treatments with the antibiotics Piperacillin/Tazobactam and Gentamicin on a mixed population of *P. aeruginosa*. We find that both treatments were able to constrain adaptation of bacteria relative to the monotherapies without resulting in increased resistance in most cases. The efficacy of evolution-informed treatments was further found to be potentiated by the antibiotic Piperacillin/Tazobactam as adaptation to it appeared to be difficult. We hypothesize that this is likely due to competitive interactions in the population. Our study provides baseline data for the success of evolution-informed treatments in a genetically diverse population of the same species.

## Introduction

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Antibiotic resistance is an evolutionary problem at its core<sup>1</sup>. Bacterial adaptation in response to the use and misuse of antibiotics in the last decades has resulted in widespread resistance, rendering them ineffective<sup>2</sup>. While this pattern of resistance evolution to antibiotics has been observed since their discovery, with the antibiotic pipeline drying up, the problem has become acute<sup>3,4</sup>. One of the solutions that have been proposed, is the use of evolutionary principles to design treatments that select against the evolution of resistance<sup>5</sup>. These so-called evolution-informed treatments have shown promise in constraining bacterial adaptation to antibiotics<sup>6–8</sup>.

One treatment strategy used to select against resistance evolution is combining multiple drugs. To be resistant to the combination of drugs, the pathogen needs to be resistant to them both. This is improbable unless the drugs share a low-cost resistance mechanism<sup>9</sup>. Combination therapy has been used successfully in the treatment of tuberculosis<sup>10</sup>, HIV<sup>11</sup> and malaria<sup>12</sup>. Certain adaptive constraints can increase the performance of combination therapy. Synergistic interactions between drugs, where the drugs potentiate each other<sup>13</sup> have been shown to increase bacterial clearance caused by combination therapy<sup>6</sup> (but also see 14, 15 and 16). Collateral sensitivity interactions, wherein resistance to a drug confers hypersensitivity to another<sup>17,18</sup>, can constrain resistance evolution when employed in drug combinations<sup>6,19,20</sup>. Resistance to each of the component drugs in monotherapy is also an important predictor of resistance evolution to the combination<sup>6,19,20</sup>. Combination therapy has also been shown to constrain the evolution of resistance by targeting heteroresistant subpopulations<sup>21</sup>.

In addition to their simultaneous combination, antibiotics can also be alternated sequentially to select against the evolution of resistance<sup>7</sup>. If the antibiotics in the sequence have independent resistance mechanisms, resistance against the first antibiotic does not confer a benefit to the second drug, resulting in selection against the resistant bacteria when the antibiotic is switched<sup>22</sup>. Like combination therapy, some adaptive constraints can enhance the efficacy of sequential therapy. Collateral sensitivity between the alternating antibiotics has been shown to constrain bacterial adaptation whether the switch happened once<sup>23–27</sup> or multiple times<sup>22,28–32</sup>. Negative hysteresis<sup>33</sup> and spontaneous resistance rates of antibiotics<sup>32</sup> have also been observed to enhance the efficacy of an antibiotic sequence. Short exposure to an antibiotic can sometimes increase the killing by another. When such negative hysteresis switches were included in sequential therapy, they reduced bacterial adaptation rate and multi-drug resistance<sup>33</sup>. Spontaneous resistance rates of an antibiotic are a measure of the likelihood that bacteria evolve resistance to it<sup>32,34</sup>. Maximizing switches to an antibiotic with low rates of spontaneous resistance in a sequence could increase bacterial clearance achieved by the treatment<sup>32</sup>. Adaptive constraints are not limited to the kind of antibiotics. The type of sequence can also impose a constraint, as the switching rate<sup>32,33</sup> and the order of the sequence<sup>30–32,35</sup> have both been observed to affect the outcome of sequential therapy.

Evolution-informed treatments have mostly been evaluated with single strains of a species<sup>6,20,23,26</sup>. However, in some infection sites, genetically distinct lineages of a species can coexist, such as in the cystic fibrosis (CF) lung<sup>36–39</sup>. The most common pathogen isolated from adult CF patients is *Pseudomonas aeruginosa*<sup>40,41</sup>. *P. aeruginosa* isolates from a single patient



vary in a number of phenotypes such as antibiotic resistance, quorum sensing, and growth profiles<sup>42,43</sup>. The diversity in phenotypes is mirrored at the genetic level, with distinct genotypes existing within a patient, sharing mutations via homologous recombination<sup>36,44</sup>. These different isolates in a patient are not in isolation, they interact resulting in the formation of a collective. Indeed, many collective behaviours have been reported in isolates from the CF lung ranging from beta-lactamase production<sup>45</sup>, production of quorum-sensing molecules<sup>46</sup>, formation of biofilms<sup>47</sup> and competition<sup>48</sup>. Such collective behaviour could accelerate resistance evolution through horizontal gene transfer<sup>49</sup> or suppress growth and thereby evolution of resistance through competitive interactions<sup>50,51</sup>. How these behaviours affect the efficacy of evolution-informed treatments is yet not known.

In this study, we evaluated sequential and combination treatments with two antibiotics, the beta-lactam and beta-lactamase inhibitor mix Piperacillin/Tazobactam (PTZ) and the aminoglycoside Gentamicin (GEN) on a mixed population of *P. aeruginosa* strains. We assessed the efficacy of a treatment regimen with multiple measures including the time taken for the bacteria to adapt, the robustness of the populations once adapted and the evolved multidrug resistance.

## Results

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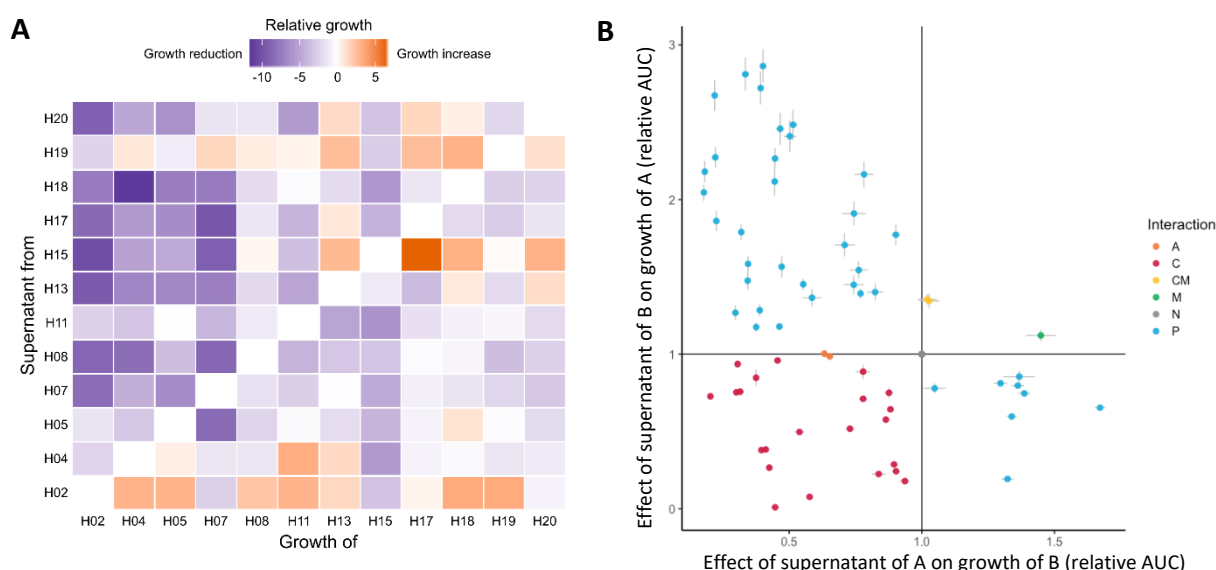
### Negative interactions dominate the mixed population

The model mixed population consisted of 12 strains of *P. aeruginosa* (Fig. S1). These strains were taken from a strain panel of the species where the constituent strains together cover the whole genomic diversity in the species<sup>52,53</sup>. Before evaluating evolution-informed treatments on this model population we wanted to assess if there existed any social interactions between them. To this aim, we performed experiments with conditioned media. Conditioned medium of strain A was used to grow strain B. The rationale was that any secreted molecules mediating social interactions would be present in this medium but not any live cells. Growth of B in the presence of these molecules would allow for the interactions to take place even though the cells producing them aren't there. As controls, we also grew strain B in media conditioned by itself. The difference in area under the growth curve (AUC) of B growing in A's conditioned medium and B growing in its own conditioned medium was used to quantify the interaction. A positive difference indicated a positive interaction and a negative difference indicated a negative interaction. We found that negative interactions dominated the interaction matrix (Fig. 1A). Of the 144 one-way interactions quantified, 69% reduced the growth of the assessed strain, 8% had no effect and 22% increased the strain's growth. We next wanted to classify the interaction between each strain pair. This was done using the method described by Kehe et al<sup>54</sup>. In short, bootstrapping was used to generate multiple sampling populations of the original AUCs. From this the ratio of AUC of B growing in A's conditioned medium and AUC of B growing in its own conditioned medium was calculated along with confidence intervals (CI95). The pairwise interactions were classified depending on if one or both interactions were less than or greater than 1.0 (Fig. 1B). In total 6 interactions were found, including: amensalism, where one strain's growth was reduced but the other strain did not show any change in growth (-,0), competition, where the growth of both strains in a pair was inhibited (-,-), commensalism, where the growth

of one strain increased but the other remained unchanged (+,0), mutualism, where both strain's growth was promoted (+,+), parasitism, where one strain's growth was promoted and the other suffered (+,-) and neutralism, where there was no change in growth for either strain (0,0). Of these, competitive and parasitic interactions were the most common.

### **Evolution-informed therapy increases the time taken for adaptation**

Having established that the strains in our mixture showed primarily competitive and parasitic interactions, we next wanted to assess if these interactions could affect the positive outcomes of evolution-informed therapy previously observed in *P. aeruginosa* strain PA14<sup>6,33,55</sup>. We carried out evolution-informed therapy with two antibiotics; PTZ and GEN. These two antibiotics were chosen because they are known to show drug synergy<sup>6</sup>, reciprocal collateral sensitivity<sup>56</sup>, and directional negative hysteresis in the direction of PTZ to GEN<sup>55</sup> in PA14 (Fig. 2A). These two antibiotics were used either sequentially or in combination resulting in three distinct evolution-informed treatments (Fig. 2B). As controls we had the single antibiotic treatments (monotherapies) and the no drug treatment. In addition to these 6 treatments, we had two meta-treatment regimens which differed in their mode of transfer to the next growth season (Fig. 2C). The no-mixing meta-treatment involved a well-to-well correspondence between the spent and fresh media plates at every transfer. This, however, can result in strain loss from the mixture due to genetic drift. To compensate for this and to encourage horizontal gene transfer (see ref <sup>57</sup>) we introduced a mixing meta-treatment. In this regimen, all the bacteria in replicate wells of a treatment were mixed together at the end of a growth season in equal proportions. The next season was inoculated from this mixture instead of corresponding wells (Fig. 2C). This gave us a hierarchical experimental design, with the different treatments nested in the meta-treatment regimens. Each treatment had a further of 8 replicates giving us a total of 96 replicate populations in the experiment. Antibiotic concentrations were chosen such that all treatments had similar initial cell densities (Fig. S2) and a diverse population left after treatment (Fig. S3). Growth over the course of the evolution experiment was monitored using optical density (Fig. 2D) and cell count (Fig. S4). The experiment lasted 15 days with the first 14 days under antibiotic selection and the last day in drug-free media to assess bacterial extinction (Fig. 2B).

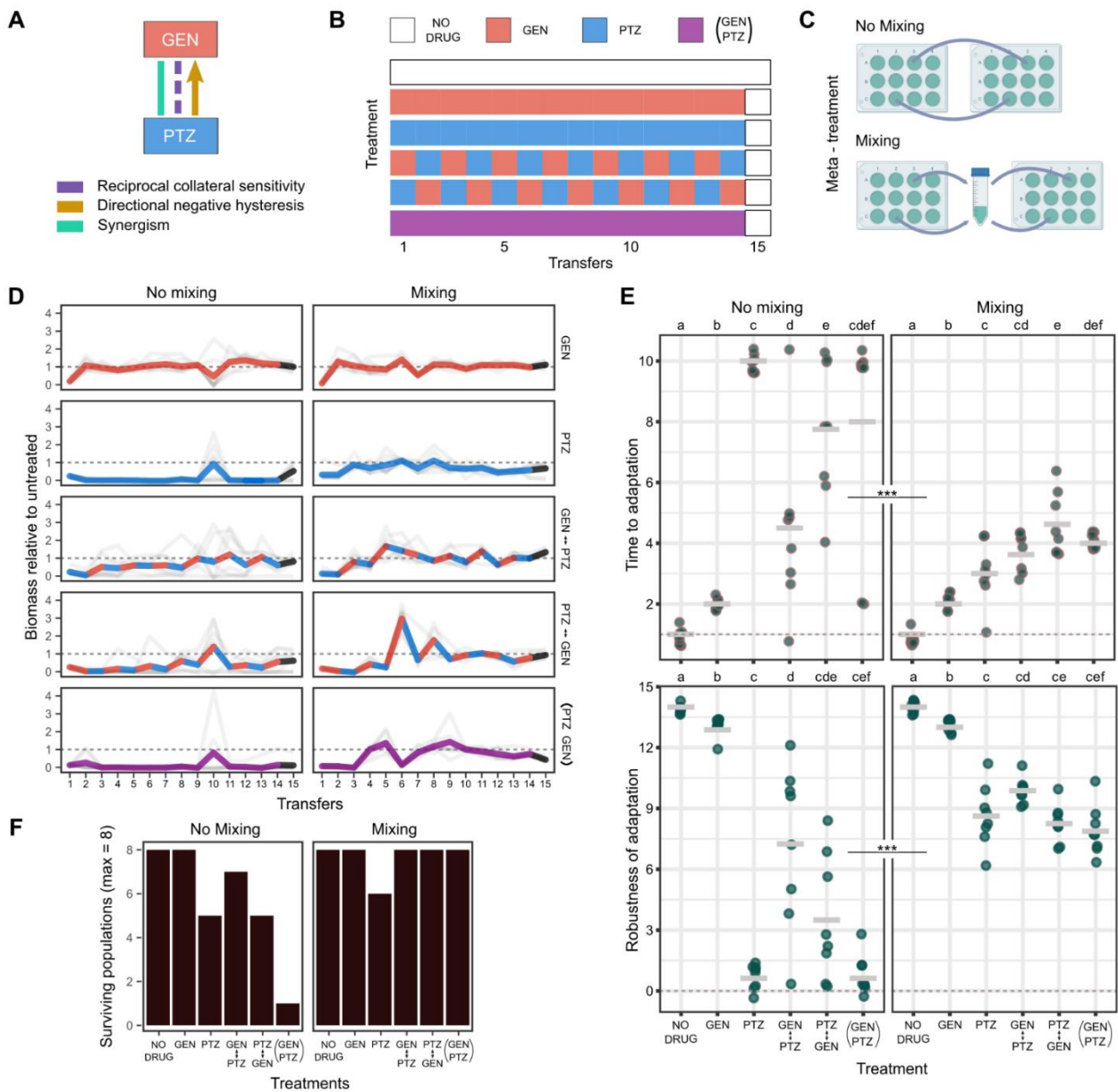


**Figure 1: Social interactions in the mixed population.**

(A) Interactions mediated by secreted molecules were assessed with the help of experiments with conditioned media. Briefly, conditioned medium from strain A was used to grow strain B. As a control, conditioned medium from strain B was also used to grow strain B. The difference in the area under the growth curve (AUC) of both gave the impact of A's conditioned medium on the growth of B. (B) Interactions in pairs of isolates. AUC obtained from growth in a second strain's conditioned medium was divided by AUC obtained from growth in self-conditioned medium and these values for each pair of strains were plotted. Based on whether these ratios were lower or higher than 1.0 on either axis the interactions were classified as (A = amensalism (-,0), C = competition (-,-), CM = commensalism (+,0), M = mutualism (+,+), N = neutralism (0,0) and P = parasitism (+,-). Error bars represent confidence intervals (CI95) calculated after bootstrapping.

We assessed adaptation to the treatment using two parameters, the time to adaptation and the robustness of the adapted populations. A population was considered adapted when it was able to grow to similar levels as the no drug control even under antibiotic selection. Consequently, the time to adaptation was the time taken for the bacterial population under selection to reach no drug levels of growth. Further, we observed zig-zag dynamics in our evolution experiment (Fig. 2D). This indicated that adaptation wasn't always stable. We calculated the number of growth seasons the bacterial population was at no drug growth levels after having adapted and used this as a measure of the robustness of adaptation. We saw that in the no-mixing meta-treatment the bacteria took significantly different times to adapt to the various treatments (Fig. 2E, randomized hierarchical ANOVA,  $F=25.76$ ,  $p<0.0001$ ). Adaptation to the GEN monotherapy was achieved in 2 days. The response to the PTZ monotherapy was very different, with the bacteria unable to grow for most of their duration under antibiotic selection. Moreover, growth was observed on the last day after the antibiotic was removed (Fig. 2D). The switching treatments were able to increase the time taken to adapt compared to the GEN monotherapy but not to the PTZ monotherapy (Pairwise Wilcoxon Rank Sum Tests,  $p<0.05$  for all). Additionally, a significant increase was observed in the switching treatment starting with PTZ relative to the one starting with GEN (Pairwise Wilcoxon Rank

Sum Tests,  $p < 0.05$ ). The response to the combination treatment was similar to the PTZ monotherapy with the bacteria unable to grow for most of their duration under antibiotic selection (Fig. 2D). Statistically, the time taken to adapt to the combination was not different from the switching treatments or the PTZ monotherapy. The robustness of adaptation showed complementary trends to that of time to adaptation. Adaptation to the GEN monotherapy was very robust with the bacteria maintaining their growth levels once adapted (Fig. 2E). PTZ treated populations were the opposite, showing only a spike in growth when the drug was removed resulting in a robustness of adaptation value of 1 (Fig. 2D and E). The switching treatments had a significantly lower value of robustness of adaptation than the GEN monotherapy (Pairwise Wilcoxon Rank Sum Tests,  $p < 0.01$ ). The GEN  $\rightarrow$  PTZ treatment had



## Figure 2: Evolution-informed treatments constrain adaptation of the mixed population.

(A) Two antibiotics, piperacillin/tazobactam (PTZ) and gentamicin (GEN) were used to evaluate the efficacy of evolution-informed treatments with a mixed population of *P. aeruginosa*. They were chosen because they display drug synergy<sup>6</sup>, directional hysteresis<sup>45</sup> and reciprocal collateral sensitivity<sup>46</sup>. (B) The antibiotics were used either sequentially or in combination. Single drug treatments and a no drug treatment served as controls. (C) The 6 treatments were nested in two meta-treatments. The meta-treatments differed in their mode of transfer to the next growth season. The no mixing meta-treatment involved a well to well correspondence during transfer after each growth season. The mixing meta-treatment involved mixing of all replicates within a treatment and inoculation of the next plate from this mixture. This was done to limit potential loss of strains due to genetic drift and to encourage horizontal gene transfer. (D) Dynamics during the evolution experiment. Biomass (OD<sup>600</sup>) relative to the no drug is plotted. Light gray line are the replicate populations, and the thicker, coloured line is the mean. Colours correspond to antibiotic treatment. n=8 per treatment. The dotted line represents no drug levels of growth. (E) Adaptation was quantified using two parameters, the time to adaptation and robustness of adaptation (see text for details). Each data point is one replicate. Bar is the mean of all replicates. (F) Surviving populations per treatment out of 8 total.

higher values than PTZ monotherapy (Pairwise Wilcoxon Rank Sum Tests,  $p < 0.01$ ) but the PTZ → GEN did not. Switching treatments starting with PTZ further resulted in a lower value of robustness of adaptation than those starting with GEN but this was not statistically significant. Lastly, the combination, like the PTZ monotherapy, was difficult to adapt to and the bacteria could only maintain their growth levels for a few seasons (Fig. 2E).

Unlike the no-mixing meta-treatment, the bacteria were able to grow in PTZ monotherapy and combination treatments in the mixing meta-treatment (Fig. 2D). Time taken to adapt in the mixing meta-treatment was significantly different to the no-mixing meta-treatment (Fig. 2E, randomized hierarchical ANOVA,  $F=65.20$ ,  $p < 0.0001$ ). Except for GEN, adaptation to all other treatments happened much faster than their no-mixing counterparts. The bacteria took a significantly longer time to adapt to PTZ monotherapy than GEN monotherapy (Pairwise Wilcoxon Rank Sum Tests,  $p < 0.01$ ). All three evolution-informed treatments increased the time to adaptation compared to the GEN monotherapy (Pairwise Wilcoxon Rank Sum Tests,  $p < 0.001$  for all). However, only the switching treatment starting with PTZ and the combination treatments were able to increase time to adaptation relative to the PTZ monotherapy (Pairwise Wilcoxon Rank Sum Tests,  $p < 0.01$  for both). The robustness to adaptation also varied significantly between the two meta-treatments (randomized hierarchical ANOVA,  $F=126.42$ ,  $p < 0.0001$ ), with the mixing meta-treatment having higher values of robustness (Fig. 2E). Bacteria were most robustly adapted to the GEN monotherapy, with all other treatments having significantly lower values of the robustness of adaptation (Pairwise Wilcoxon Rank Sum Tests,  $p < 0.001$  for all). The two switching treatments also varied in their robustness values with the PTZ → GEN treatment being less robust (Pairwise Wilcoxon Rank Sum Tests,  $p < 0.01$ ). The combination treatment was less robust than the GEN → PTZ treatment (Pairwise Wilcoxon Rank Sum Tests,  $p < 0.01$ ) but not the PTZ → GEN treatment. There was no difference observed between the other treatments in this parameter.

Some bacterial populations went extinct during the course of the experiment (Fig. 2F). Extinction was more frequent in the no-mixing meta-treatment than in the mixing meta-

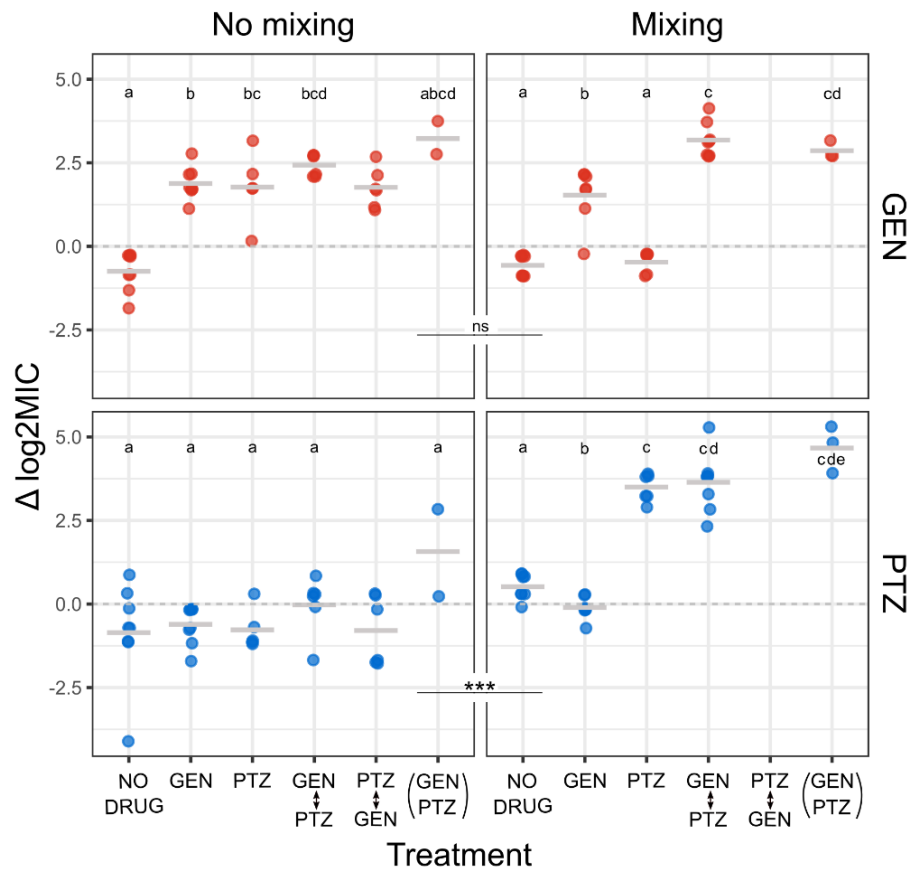
treatment (35% and 5% respectively). This is likely due to the rescue of poorly performing populations by mixing them with better performing replicates of the same treatment. Extinctions always occurred in populations that had been treated with PTZ at some point in the experiment. There were more extinctions in treatments that either started with PTZ or had a constant supply of PTZ throughout.

Overall, we conclude that evolution-informed treatments can increase the time taken to adapt by a mixed population of *P. aeruginosa* compared to monotherapy. Furthermore, we find that the efficacy of evolution-informed treatments is likely potentiated by PTZ, as adaptation to PTZ appears to be difficult. We also find that the meta-treatment affects the ability of bacteria to adapt, with the mixing meta-treatment resulting in faster adaptation than the no-mixing meta-treatment.

### **Resistance evolution to PTZ is limited in the no-mixing meta-treatment**

We next wanted to test if the evolution-informed treatments led to a decrease in the evolved resistance of the bacterial populations. We calculated the fold change in minimum inhibitory concentrations (MIC) of populations recovered from season 14 on GEN and PTZ using Etests<sup>58</sup>(Fig. 3). We found that there was no significant difference in GEN resistance between the no mixing and mixing meta-treatments. Within a meta-treatment however, the treatments varied in their GEN resistance (randomized hierarchical ANOVA,  $F=49.52$ ,  $p<0.0001$ ). In the no mixing meta-treatment, resistance evolution to GEN happened in all treatments relative to the no drug except the combination treatment (Pairwise Wilcoxon Rank Sum Tests,  $p<0.05$  for all except no drug – combination comparison). Bacterial populations treated only with PTZ evolved cross-resistance to GEN. Furthermore, none of the evolution-informed treatments resulted in higher resistance than the monotherapies. In the mixing treatment, resistance evolution happened in all treatments except the PTZ monotherapy (Pairwise Wilcoxon Rank Sum Tests,  $p<0.05$  for all except no-drug-PTZ comparison). The PTZ monotherapy had resistance levels similar to the ancestor (Fig. 3). The evolution-informed treatments in the mixing meta-treatment had significantly higher GEN resistance than the monotherapies (Pairwise Wilcoxon Rank Sum Tests,  $p<0.05$ ). Unlike GEN resistance, resistance to PTZ varied significantly between the meta-treatments (randomized hierarchical ANOVA,  $F=139.589$ ,  $p<0.0001$ ). In the no mixing meta-treatment, no evolution of PTZ resistance was observed (Fig. 3). All treatments had similar levels of resistance as the no drug control. The mixing meta-treatment selected for resistance to PTZ but not in all treatments. The GEN monotherapy had lower resistance than no drug treatments on PTZ (Pairwise Wilcoxon Rank Sum Tests,  $p<0.05$ ), suggestive of collateral sensitivity interactions like those observed in PA14<sup>56</sup>. Resistance was observed on the PTZ monotherapy and on the evolution-informed treatments but was not significantly different between them. Similar trends were also observed when a different measure of resistance ( $\Delta AUC$ ) was calculated using broth microdilution (Fig. S6). To conclude, we find that resistance evolution to PTZ is constrained in the no-mixing

meta-treatment. Further, though evolution-informed treatments result in resistance evolution in other cases, it is mostly not more than monotherapies.



**Figure 3: MIC fold change of evolved populations on PTZ and GEN**

The log<sub>2</sub>MIC fold change relative to the ancestral mixed population is plotted. Frozen populations from season 14 were regrown and the MIC was measured using Etests. The number of recovered populations varied from 2-8 per treatment. No populations were recovered for the PTZ → GEN treatment in the mixing meta-treatment. Dots are the individual replicate populations while the bar is the mean. The dotted line represents the ancestral log<sub>2</sub>MIC. The plot is split according to the two meta-treatments given at the top, and the antibiotics given on the right. The treatments are given at the bottom. A significant difference was seen between the meta-treatments for resistance to PTZ only (randomized hierarchical ANOVA,  $F=139.58$ ,  $p<0.0001$ ). Evolved resistance between treatments varied in both meta-treatments (randomized hierarchical ANOVA,  $F= 49.52$  and  $20.50$  for PTZ and GEN respectively,  $p<0.0001$  for both). PTZ: Piperacillin/Tazobactam, GEN: Gentamicin.

## Discussion

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Evolution-informed treatments offer sustainable alternatives to the current standard of care as they can constrain adaptation in bacteria<sup>5</sup>. They have, to date, only been evaluated in single strains. Cooperative and competitive interactions between bacteria, such as those found between *P. aeruginosa* strains in the CF lung can potentially impact the outcome of evolution-informed treatments. In this study, we assessed the ability of two evolution-informed treatments, sequential and combination treatments with the antibiotics PTZ and GEN, to constrain adaptation in a mixed population of *P. aeruginosa* strains. We found that the evolution-informed treatments increased the time the bacteria took to adapt without resulting in higher resistance than the monotherapies in most cases. Their efficacy was potentiated by the antibiotic PTZ, as adaptation to PTZ appeared to be difficult. Adaptation was further found to be influenced by the meta-treatment used for transfer.

Why was adaptation to PTZ more difficult? In earlier studies from our lab, strain PA14 of *P. aeruginosa* adapted to PTZ monotherapy, although slower than on other antibiotics<sup>6,56</sup>. Even then, in the no mixing conditions, the slow speed of adaptation was striking. Perhaps the presence of other strains plays a role. Community context has been shown to select against the *de novo* evolution of traits in different environments<sup>59,60</sup>, including antibiotics<sup>50</sup>. Further, the presence of a community has been observed to increase the fitness costs of existing resistance genes<sup>51</sup>. These community-based effects were mediated by competitive interactions<sup>51,59,60</sup>. It is plausible that the competitive interactions between the mixed population constrained adaptation on PTZ. However, why would adaptation only be constrained on PTZ? The cost of antibiotic resistance has been previously observed to vary between different antibiotics<sup>51</sup>. This was due to one of the treatments enriching for bacteria more closely related to the focal strain thereby increasing resource competition<sup>51</sup>. It could happen that the composition of the mixed population varied between the different antibiotics resulting in more competitive interactions on PTZ. Yet, the competition should have been fiercer in the mixing meta-treatment as it was aimed at preventing strain loss. Instead, adaptation was sped up there. This makes competition mediated by strain composition less likely to be the cause. Fitness costs of resistance mutations vary by drug class<sup>61</sup>. It could be that low fitness cost mutations on PTZ are limited. In a previous study from our lab, PA14 had higher costs due to PTZ resistance than GEN<sup>56</sup>. The cost of easily available high cost mutations is heightened by intraspecies competition making it difficult for those bacteria to survive. This could select alternative strategies for survival like bacterial tolerance<sup>58</sup>. Indeed, bacteria in the PTZ monotherapy grew once the antibiotic was removed, suggesting tolerance behaviour<sup>62</sup>. Switching to alternative strategies to avoid higher costs imposed by competition has been reported in *P. aeruginosa* before. Interspecific competition resulted in a switch from costly receptor-based phage resistance to the less costly CRISPR-based resistance<sup>63</sup>. The mixing meta-treatment increases bacterial mutational supply by decreasing extinction. This increase in mutational supply could have made available rarer low-cost mutations on PTZ resulting in adaptation. Another possibility is that the mixing meta-treatment reduced strain loss and encouraged horizontal gene transfer resulting in the fast spread of resistance via a mobile genetic element. The genomic analysis of these populations would serve as a first step in testing these hypotheses.



We found that evolution-informed treatments with PTZ and GEN were able to constrain adaptation in a mixed population of *P. aeruginosa*. The impact of social interactions in a community on resistance evolution has been studied before, albeit mostly in antibiotic monotherapies. The outcome of resistance evolution depends on the exact social interactions occurring in the community of choice. Cross-feeding was observed to slow down *de novo* resistance evolution<sup>64,65</sup>. This was because the increase in resistance was always in steps compared to the monocultures; one partner always had to ‘wait’ for its cross-feeding partner to evolve higher resistance for the coculture to survive an increase in antibiotic concentration<sup>64</sup>. The presence of antibiotic-degrading enzymes had a beneficial effect on resistance evolution for both, the producers of the enzyme<sup>66</sup> and the surrounding non-producers<sup>67</sup>. Exposure to ampicillin increased the frequency of  $\beta$ -lactamase producing *E. coli*, proportional to the antibiotic concentration divided by the cell density<sup>66</sup>. In another study, co-culture of *P. aeruginosa* with carbapenemase-producing *Stenotrophomonas maltophilia* resulted in accelerated *de novo* evolution of imipenem resistance in *P. aeruginosa*<sup>67</sup>. This was likely because protection provided by the carbapenemase allowed for higher cell densities, increasing the mutational supply<sup>67</sup>. Competitive interactions have a negative effect on the evolution of resistance. Interspecific competition was found to increase the costs of resistance genes in a focal *E. coli* strain resulting in higher minimal selective concentrations<sup>51</sup>. However, when the concentration of the antibiotic was increased, it led to reduced cell numbers in the community and competitive release of the focal strain<sup>51</sup>. These dynamics appear to be universal as other studies have also reported restricted resistance evolution, both *de novo* and from standing genetic variation in the presence of multispecies communities<sup>50,65,68</sup>. A recent study assessed single and combined antibiotic treatments on co-cultures of *P. aeruginosa* and *S. maltophilia* as they frequently co-infect CF patients. They found that antibiotic resistance evolved in all treatments but the exact composition of the community varied with the treatment. Usually, the more resistant of the species to the antibiotic in question dominated<sup>69</sup>. Our study demonstrates that interactions in a mixed population of a single species can also affect resistance evolution. It further confirms the efficacy of evolution-informed treatments in a mixed population dominated by negative interactions.

To conclude, our study shows that switching and combination treatments with PTZ and GEN could constrain adaptation in a model mixed population of *P. aeruginosa*. The generality of our findings, however, needs to be tested. Our model population had a lot of negative interactions, which are known to hinder adaptation. Evolution-informed treatments should be evaluated with populations displaying more positive interactions. Further, we used PTZ and GEN because they displayed adaptive constraints. More antibiotics, with or without these constraints should be tested. We used a mixed population of *P. aeruginosa*, but infections can also be due to multi-species communities. It would be interesting to extend our setup to such a community. Lastly, we have only speculated about the possible reasons adaptation to PTZ was difficult. The exact mechanism needs to be delineated and will be the focus of future experiments.

## Materials and Methods

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### Strains

The mixed population used for experiments consisted of 12 strains of *P. aeruginosa*. These were taken from a panel consisting of strains representing the entire genomic diversity of the species (Fig. S1)<sup>52,53</sup>. Each of the 12 strains was grown separately in 10mL Luria Bertani (LB) medium for 20h at 37°C and 150 RPM following which they were mixed in a 1:1 ratio (v/v) to obtain the final mixed population. No statistically significant difference in the CFU/mL was observed at the end of 20h between the different strains (Kruskal Wallance Test,  $\chi^2=19$ ,  $p=0.456$ ) This mixture was then used for experiments.

### Media and antibiotics

All experiments with the mixed population were conducted in either Luria Bertani (LB) or minimal M9 medium or agar (1.5%). M9 medium was supplemented with glucose (2 g/L), citrate (0.58 g/L), and casamino acids (1 g/L). Antibiotics were added when required. Antibiotics used included the aminoglycoside Gentamicin (GEN, Carl Roth, Order No. HN09.1) and a combination of the beta-lactam Piperacillin (PIP, Sigma Aldrich, Code: P8396-1G) and the beta-lactamase inhibitor Tazobactam (TAZ, Sigma Aldrich, Code: T2820-10MG). Piperacillin and Tazobactam (PTZ) were combined in an 8:1 ratio. Bacteria were incubated at 37°C.

### Experiments with conditioned media

To determine contact-independent pairwise interactions, we cultured the 12 strains in LB and M9 media for 24hr at 37 °C temperature. After incubation, cultures grown in the M9 medium were filtered through 0.22  $\mu$ m filters to generate bacteria-free supernatant. Strains grown in LB were sub-cultured until the mid-exponential phase and washed from the residual medium. We resuspended washed bacteria in the filtered M9 medium using the matrix-like format in a 96-well plate and normalized the initial OD at OD<sub>600</sub> = 0.001. Cultures were incubated at 37 °C for 31hr with shaking (162 RPM). Measurements of OD<sub>600</sub> were taken every 15 minutes with a microplate reader (Tecan, Spark). The OD<sub>600</sub> values were normalized by subtracting media-only control. We performed 3 independent biological repetitions for each combination of strain and conditioned media. Area under curve (AUC) measures were calculated using the R package *growthcurver*. Raw AUC values were bootstrapped to obtain sampling populations from which the ratio of AUC of B growing in A's conditioned medium and B growing in self-conditioned medium was calculated. Confidence intervals (CI95) were computed as a measure of uncertainty (see ref <sup>54</sup>). From these ratio's, pairwise interactions were classified depending on if one or both interactions were less than or greater than 1.0.

### CFU- based dose-response curves

Dose-response curves (DRC) were carried out to determine the concentration of antibiotics reducing cell number by three orders of magnitude compared to the no drug control. Individual strains were grown overnight for 20h in LB and then mixed in a 1:1 (v/v) ratio. 40  $\mu$ L of this mixture was inoculated into the wells of 12 well plates containing a range of antibiotic

concentrations chosen based on the minimum inhibitory concentration (MIC) data of the individual strains. No drug controls and blanks were included. All treatments were randomized on the plate. Total volume in each well was 2000  $\mu$ L. Plates were incubated at 37°C and 900 RPM for 24h. At the end of incubation, the plates were sonicated for 1 min to break any clumps of bacteria following which bacteria were spotted to determine CFU/mL. This was done for GEN, PTZ, and their combination (Fig. S2). For the combination DRC, each concentration tested contained an equal mixture of the two antibiotics.

### Strain-specific Polymerase Chain Reaction (PCR)

To detect which strains survived post-antibiotic treatment, strain specific PCRs were carried out. Each strain was uniquely identified by a primer pair (for a list of primer pairs see Table S13). Samples were taken at concentrations that reduced the cell number by three orders of magnitude and subjected to PCRs. The PCR reaction mix and program used were as follows:

#### *Reaction mixture*

Volume (20 $\mu$ L reaction)	Component
13.2 $\mu$ L	dH <sub>2</sub> O
2.0 $\mu$ L	10x Dream Taq buffer
2.5 $\mu$ L	dNTPs (2mM)
1.0 $\mu$ L	Forward primer (10mM)
1.0 $\mu$ L	Reverse primer (10mM)
1.0 $\mu$ L	Template DNA
0.3 $\mu$ L	Taq polymerase

#### *PCR Program*

Temperature	Time	Cycles
95 °C	5 min	
95 °C	45 s	} 30 x
55 °C	30 s	
72 °C	30 s	
72 °C	10 min	

After 24 h of growth under antibiotic the strain number reduced from 12 to 4 and 7 on PTZ and GEN, respectively (Fig. S3).

### Evolution experiment

The evolution experiment consisted of 6 treatments nested within 2 meta-treatments. The 6 treatments included 3 evolution-informed treatments (one combination and two sequential, each starting with a different antibiotic) and 3 controls (No drug, monotherapy with each antibiotic). Each treatment contained 8 replicate populations. Treatments were fully randomized on each plate. Each of the 12 strains were grown for 20h in LB and then mixed together in a 1:1 (v/v) ratio. From independent mixtures bacteria were inoculated into 12 well antibiotic plates containing 2000  $\mu$ L of medium. They were then grown for 24 h at 37°C and 900 RPM, following which the plates were sonicated to break any bacterial clumps. 40 $\mu$ L (2% v/v) was then transferred to another plate containing fresh medium. When there was a 1-to-1 correspondence between wells during transfer this was termed the 'No mixing' meta-treatment. The 'mixing' meta-treatment was introduced to limit strain loss due to genetic drift. In this, all

8 replicates within a treatment were mixed together in one Falcon tube and this mixture was used to inoculate all wells of this treatment for the next growth season. Each of the meta-treatments had 6 treatments making a total of 12 treatments and 96 bacterial populations. The experiment lasted a total of 15 days with 14 days of antibiotic exposure followed by 1 day of growth in drug free medium to assess population extinction and fitness costs. Populations were frozen in DMSO on days 4, 7, 11 and 14. Populations which grew after removal of antibiotic were frozen on day 15.

### **Calculation of population extinction and adaptation parameters**

Both population extinction and adaptation of the surviving bacterial populations to the treatment was quantified at the end of the evolution experiment. A population was said to be extinct if in the last growth season without drug its  $OD_{600}$  was less than 0.05. Two parameters were used for quantifying adaptation. Time to adaptation is the time taken (in number of growth seasons) for the bacterial population under treatment to attain the same level of growth as those which do not see any drug. This gives us an idea of the potential of a treatment to retard bacterial adaptation. Robustness of adaptation is the number of growth seasons the bacterial population under treatment is able to stay adapted. This is a measure of how well adaptation was maintained once acquired. A population under treatment is said to grow at the same level as that under the no drug regimen if its  $OD_{600}$  was within the mean  $\pm$  2SD of the no drug population in that growth season.

### **Minimum inhibitory concentration (MIC) measurement using Etest**

The MIC of both antibiotics for all evolved populations and the ancestral population was measured using the Epsilonometer Test or Etest (Liofilchem)<sup>58</sup>. For the ancestral population, the 12 strains were grown individually in 10 mL LB for 20h, mixed together in a 1:1 ratio. This mixture was then diluted to an  $OD_{600}$  of 0.08 and spread onto a M9 plate using a cotton swab. Etest strips (Liofilchem) were placed onto the plate and the plate incubated for 24h after which the MIC was read at the intersection of the zone of inhibition and the test strip for GEN. For PTZ, we observed bacterial growth in the shape of wings above the intersection of the zone of inhibition and the test strip (Fig. S5). This is likely the paradoxical effect<sup>70</sup>. It is important to note that this effect was not observed on PTZ when individual strains were tested. The MIC was read where the top edge of the wings intersected the test strip. The frozen populations from day 14 were thawed and 50  $\mu$ L inoculated into 10 mL LB. These were grown for 20h at 37°C and 150 RPM. After incubation, the cultures were diluted to an  $OD_{600}$  of 0.08 and then spread onto a plate with a swab. Etest strips (Liofilchem) were placed onto the plate and incubated for 24h after which the MIC was read either at the intersection of the zone of inhibition and the strip or where the top edge of the wings intersected the strip. Change in MIC of the evolved population was calculated as  $MIC_{\text{evolved population}} - MIC_{\text{ancestor}}$

### **OD-based dose-response curves**

Etests measure antimicrobial susceptibility based on growth on agar. In order to have a measure of antimicrobial susceptibility based on growth in liquid media, we carried out OD-based dose-response curves. For the ancestral population, the 12 strains were grown individually in 10 mL LB for 20h after which they were mixed together in a 1:1 ratio. 2  $\mu$ L of this mixture was

inoculated into the wells of a 96-well plate (100  $\mu$ L total volume) containing a range of concentrations of antibiotics covering the MICs of the individual strains. The plate was incubated at 37°C and 750 RPM for 24 h after which the OD<sub>600</sub> was read in a BioTek plate reader. The area under the resulting dose-response curve was calculated using the *drc* package in R<sup>71</sup> and was used as a measure of the antimicrobial susceptibility of the ancestral population. For measuring the antimicrobial susceptibility of the evolved populations, the populations frozen on day 14 were thawed and 50  $\mu$ L was inoculated into 10 mL LB. The cultures were incubated at 37°C and 150 RPM for 20h. Afterwards, 2  $\mu$ L of the culture was inoculated into the wells of a 96-well plate (100  $\mu$ L total volume) containing a range of concentrations of antibiotics centred around the MIC of the ancestral population. The plate was incubated at 37°C and 750 RPM for 24 h after which the OD<sub>600</sub> was measured. The area under the resulting dose-response curve was calculated and used as a measure of the antimicrobial susceptibility of the evolved population. Change in antimicrobial susceptibility of the evolved population was calculated as antimicrobial susceptibility<sub>evolved population</sub> - antimicrobial susceptibility<sub>ancestor</sub>.

### Statistical analysis

Statistical analysis was carried out in R version 4.0.3 using packages *car* and *lawstat*. The response variables ‘Time to adaptation’, ‘Robustness of adaptation’, ‘MIC on GEN’, ‘MIC on PTZ’, ‘Antimicrobial susceptibility on GEN’, and ‘Antimicrobial susceptibility on PTZ’ were analyzed with the same statistical framework. A nested randomization Analysis of Variance (ANOVA) was used to assess the impact of the predictor variables ‘Meta-Treatment’ and ‘Treatment’ on the response variables. The variable ‘Treatment’ was nested in the variable ‘Meta-Treatment’. The general model of the ANOVA used was *aov(Response ~ Meta.Treatment/Treatment, data = datadf)*. The model was run 10000 times with the data randomized at each run to generate a distribution of F ratios expected under the null hypothesis of no association. P value was calculated as the probability of obtaining F ratios greater than or equal to the F ratios observed with the original data. Post hoc testing was carried out using pairwise Wilcoxon rank sum tests.

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### Competing interests statement

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The authors declare no competing interests.

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### EVOLUTION-INFORMED TREATMENTS CONSTRAIN ADAPTATION IN A GENETICALLY DIVERSE POPULATION OF *PSEUDOMONAS AERUGINOSA* SUPPLEMENTARY INFORMATION

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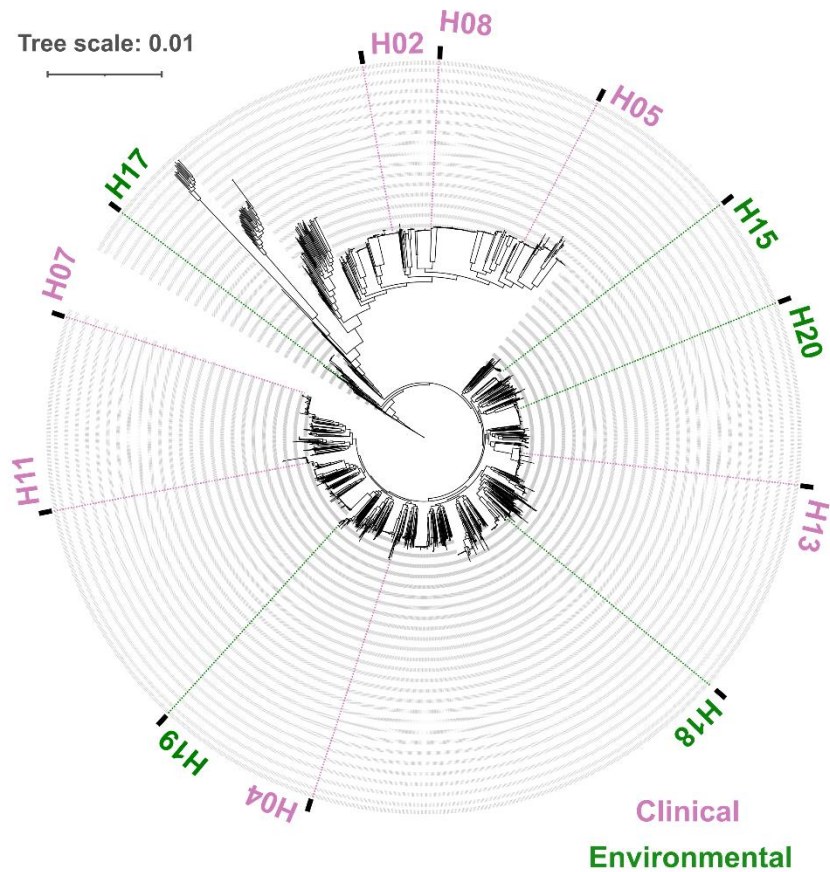
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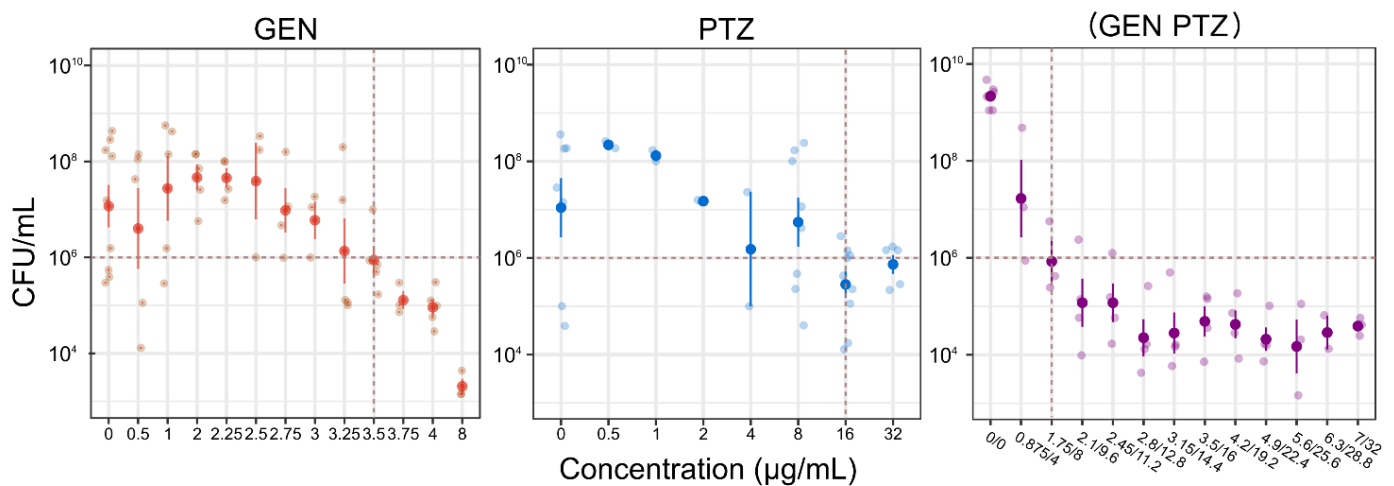
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**Figure S1: Strains constituting the mixed population**

The mixed population consisted of 12 strains of the species *P. aeruginosa*. The positions of these strains on a phylogeny of all published *P. aeruginosa* genomes is depicted (Figure modified from ref 1). These strains are derived from a strain panel of the species<sup>1</sup> and are phylogenetically distinct.

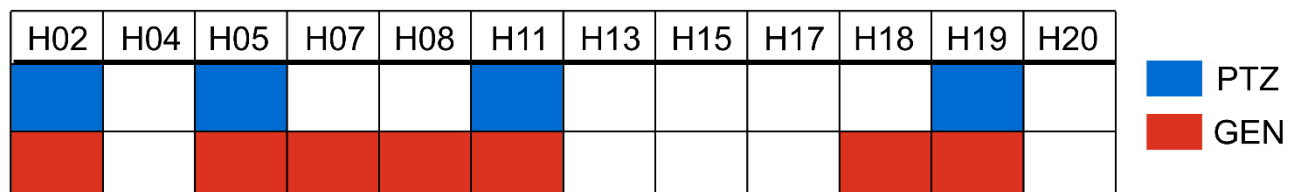


**Figure S2: Dose-response curves to determine concentrations to be used in the evolution experiment.**

Dose-response curves were carried out to determine the concentration of the antibiotics, singly and in combination, which reduced the cell number to  $10^6$  cells/mL. These concentrations were then used in the evolution experiment with the aim of equalizing the initial cell density.

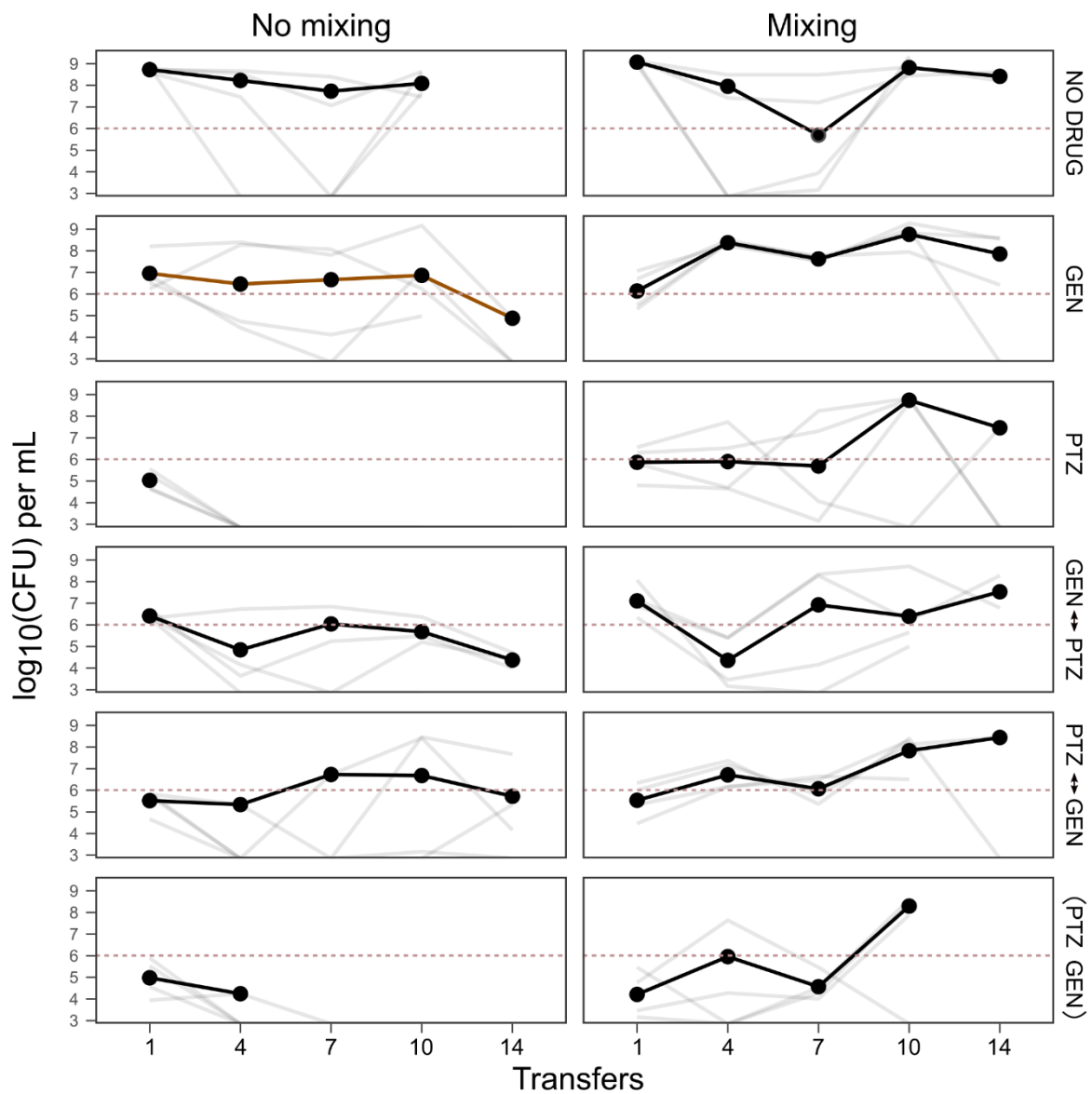
**Supplementary Table S1. Strain-specific primers to detect individual strains of the mixed population**

Primer	Name	Sequence
H02_forward	H02_P1_F	GGA CGG TTG CCA CAA TGT TC
H02_reverse	H02_P1_R	CTT GCC GTT GAT AGC GAT GC
H04_forward	H04_P1_F	CGT TCA GCG CTA CTT CAT CG
H04_reverse	H04_P1_R	CTC CTG CTC TGG TTT CCT GG
H05_forward	H05_P1_F	CGC TGC TAC TCT CCA TCC TG
H05_reverse	H05_P1_R	CTG AGG CCT GGC TGA TGT AG
H07_forward	H07_P1_F	ACT GAA CGT AGC CAT GGA GC
H07_reverse	H07_P1_R	CGC CAG GTT TAG AGC CAG AG
H08_forward	H08_P1_F	CTG ATG CCG GAG ATC ACC TG
H08_reverse	H08_P1_R	GTG AGC GGT TCC AGG GAT G
H11_forward	H11_P1_F	GGC CGT ATC CTT TAC CTC CG
H11_reverse	H11_P1_R	ATC AGC AGT TCG TCA GCC TC
H13_forward	H13_P1_F	ACG GCA GTC TCC ATG AGT TC
H13_reverse	H13_P1_R	GCT CCG ATA CCG TAG AAG CC
H15_forward	H15_P1_F	AGA TGT TCC AGA AGC ZGC GC
H15_reverse	H15_P1_R	GAG AGT TTG CGT CTA TGT GC
H17_forward	H17_P1_F	GAG CGA CGA ATT CCT GCA AC
H17_reverse	H17_P1_R	GAC CAT CTC CAC TAG CTC GC
H18_forward	H18_P1_F	CCG CAG CCT ATC TCT ACA CG
H18_reverse	H18_P1_R	CCG GGT AGA GTT GGA TCA CG
H19_forward	H19_P1_F	TTG ACG ATC CTC AGG TGC TG
H19_reverse	H19_P1_R	CGA GGT GAT CTG CGA CTA CC
H20_forward	H20_P1_F	CCG GAC AGT TAC ACC TCG TC
H20_reverse	H20_P1_R	TCG GCG TCA TGT GTC AGT AC



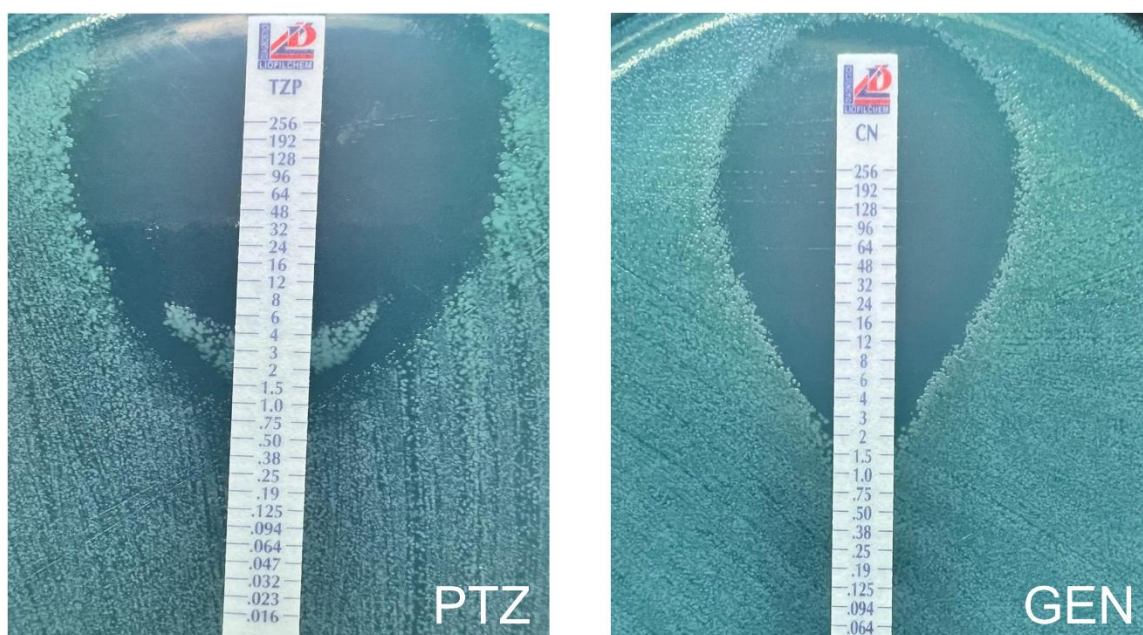
**Figure S3: Strain diversity remaining after 24h under antibiotic treatment.**

To assess the diversity of the mixed population after the initial antibiotic treatment we carried out strain-specific PCRs from the antibiotic treated mixture. Genetic diversity was found to be reduced after antibiotic treatment but not eliminated.



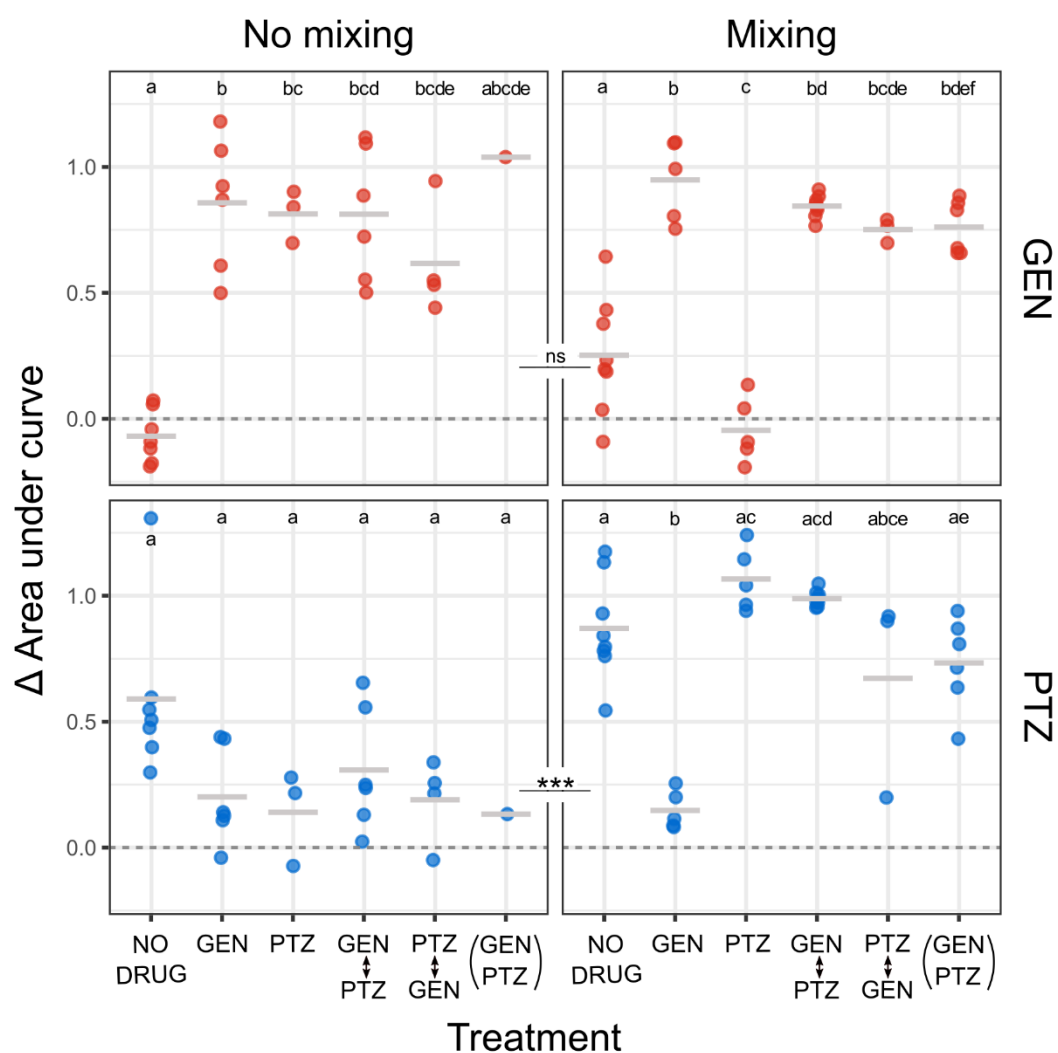
**Figure S4: Cell count across treatments in the evolution experiment.**

Growth of the mixed population was monitored over the course of the evolution experiment using OD<sub>600</sub> measurements as well as cell counts. The cell counts, though noisy, showed similar patterns of growth to the OD<sub>600</sub> measurements.



**Figure S5: Antimicrobial susceptibility testing with Etest of the ancestral mixed population.**

The antimicrobial susceptibility of the ancestral mixed population on agar was determined using Etests. The Etest for PTZ displayed a wing-like bacterial growth above the intersection of the zone of inhibition and the test strip. This is reminiscent of the paradoxical or Eagle effect<sup>3</sup>. This effect was not observed in the single strains. MIC was read for PTZ where the upper part of the wings intersected the test strip (4 $\mu$ g/mL) and where the zone of inhibition intersected the test strip (3 $\mu$ g/mL) for GEN.



**Figure S6: Antimicrobial susceptibility (Area under the dose-response curve) of the evolved populations.**

The area under the dose-response curve (AUC) for PTZ and GEN was measured, normalized to the ancestral population, and plotted. Frozen populations from season 14 were regrown and subjected to broth microdilution. The number of recovered populations varied from 2-8 per treatment. Dots are the individual replicate populations while the bar is the mean. The dotted line represents the ancestral AUC. The plot is split according to the two meta-treatments given at the top, and the antibiotics given on the right. The treatments are given at the bottom. The results are similar to those from MIC measurements. A significant difference was seen between the meta-treatments for resistance to PTZ only (randomized hierarchical ANOVA,  $F=75.569$ ,  $p<0.0001$ ). Evolved resistance between treatments varied in both meta-treatments (randomized hierarchical ANOVA,  $F= 8.342$  and  $27.26$  for PTZ and GEN respectively,  $p<0.0001$  for both). PTZ: Piperacillin/Tazobactam, GEN: Gentamicin.

**Supplementary Table S2. ANOVA table for the time taken till adaptation during the evolution experiment <sup>a</sup>**

Source of variation	Sums of squares	<i>df</i>	<i>F-ratio</i>	<i>P</i>
Meta treatment	150.0	1	65.20	< <b>0.0001</b>
Meta treatment: Treatment	592.6	10	25.76	< <b>0.0001</b>
Residuals	193.2	84		

<sup>a</sup> The effects of the two factors, Meta-treatment and Treatment on the response variable time to adaptation was assessed using a nested permutation ANOVA (Treatment nested in Meta-treatment). The analysis was performed in R version 4.0.3 using the model *anova(aov(Time.to.adaptation ~ Meta.Treatment/Treatment, data = Adaptdf)*. 10000 runs of permutation were carried out. Significant *P* values are indicated in bold.

**Supplementary Table S3. Post hoc tests comparing Treatments within a Meta-treatment for time taken till adaptation during the evolution experiment <sup>a</sup>**

Comparison	<i>Adjusted P</i>
<b>No mixing</b>	
Drug free – Monotherapy GEN	<b>0.00069</b>
Drug free – Monotherapy PTZ	<b>0.00069</b>
Drug free – (GEN -> PTZ)	<b>0.00261</b>
Drug free – (PTZ -> GEN)	<b>0.00096</b>
Drug free – (PTZ + GEN)	<b>0.00096</b>
Monotherapy GEN – Monotherapy PTZ	<b>0.00069</b>
Monotherapy GEN – (GEN -> PTZ)	<b>0.01217</b>
Monotherapy GEN – (PTZ -> GEN)	<b>0.00096</b>
Monotherapy GEN – (PTZ + GEN)	<b>0.00551</b>
Monotherapy PTZ – (GEN -> PTZ)	<b>0.00261</b>
Monotherapy PTZ – (PTZ -> GEN)	<b>0.01716</b>
Monotherapy PTZ – (PTZ + GEN)	0.18209
(GEN -> PTZ) – (PTZ -> GEN)	<b>0.02764</b>
(GEN -> PTZ) – (PTZ + GEN)	0.12714
(PTZ-> GEN) – (PTZ + GEN)	0.45070



<b>Mixing</b>	
Drug free – Monotherapy GEN	<b>0.00069</b>
Drug free – Monotherapy PTZ	<b>0.00221</b>
Drug free – (GEN -> PTZ)	<b>0.00069</b>
Drug free – (PTZ -> GEN)	<b>0.00069</b>
Drug free – (PTZ + GEN)	<b>0.00069</b>
Monotherapy GEN – Monotherapy PTZ	<b>0.00992</b>
Monotherapy GEN – (GEN -> PTZ)	<b>0.00069</b>
Monotherapy GEN – (PTZ -> GEN)	<b>0.00069</b>
Monotherapy GEN – (PTZ + GEN)	<b>0.00069</b>
Monotherapy PTZ – (GEN -> PTZ)	0.12551
Monotherapy PTZ – (PTZ -> GEN)	<b>0.00520</b>
Monotherapy PTZ – (PTZ + GEN)	<b>0.00573</b>
(GEN -> PTZ) – (PTZ -> GEN)	<b>0.02652</b>
(GEN -> PTZ) – (PTZ + GEN)	0.08141
(PTZ-> GEN) – (PTZ + GEN)	0.08141

<sup>a</sup> Post hoc comparisons following a significant nested permutation ANOVA were carried out using Pairwise Wilcoxon Rank Sum Tests in R version 4.0.3. *P* values were adjusted for multiple comparisons using the False Discovery Rate (FDR) method: Significant *P* values are given in bold.

**Supplementary Table S4. ANOVA table for the robustness of adaptation during the evolution experiment <sup>a</sup>**

Source of variation	Sums of squares	df	F-ratio	P
Meta treatment	345.04	1	126.427	< <b>0.0001</b>
Meta treatment: Treatment	1676.21	10	61.418	< <b>0.0001</b>
Residuals	229.25	84		

<sup>a</sup> The effects of the two factors, Meta-treatment and Treatment on the response variable robustness of adaptation was assessed using a nested permutation ANOVA (Treatment nested in Meta-treatment). The analysis was performed in R version 4.0.3 using the model *anova(aov(Robustness.of.adaptation~ Meta.Treatment/Treatment, data = Adaptdf))*. 10000 runs of permutation were carried out. Significant *P* values are indicated in bold.

**Supplementary Table S5. Post hoc tests comparing Treatments within a Meta-treatment for robustness of adaptation during the evolution experiment <sup>a</sup>**

Comparison	Adjusted P
<b>No mixing</b>	
Drug free – Monotherapy GEN	<b>0.00095</b>
Drug free – Monotherapy PTZ	<b>0.00095</b>
Drug free – (GEN -> PTZ)	<b>0.00095</b>
Drug free – (PTZ -> GEN)	<b>0.00095</b>
Drug free – (PTZ + GEN)	<b>0.00095</b>
Monotherapy GEN – Monotherapy PTZ	<b>0.00095</b>
Monotherapy GEN – (GEN -> PTZ)	<b>0.00107</b>
Monotherapy GEN – (PTZ -> GEN)	<b>0.00102</b>
Monotherapy GEN – (PTZ + GEN)	<b>0.00095</b>
Monotherapy PTZ – (GEN -> PTZ)	<b>0.00964</b>
Monotherapy PTZ – (PTZ -> GEN)	0.05528
Monotherapy PTZ – (PTZ + GEN)	0.55569
(GEN -> PTZ) – (PTZ -> GEN)	0.07723
(GEN -> PTZ) – (PTZ + GEN)	<b>0.00733</b>
(PTZ-> GEN) – (PTZ + GEN)	0.05528

<b>Mixing</b>	
Drug free – Monotherapy GEN	<b>0.00065</b>
Drug free – Monotherapy PTZ	<b>0.00065</b>
Drug free – (GEN -> PTZ)	<b>0.00065</b>
Drug free – (PTZ -> GEN)	<b>0.00065</b>
Drug free – (PTZ + GEN)	<b>0.00065</b>
Monotherapy GEN – Monotherapy PTZ	<b>0.00065</b>
Monotherapy GEN – (GEN -> PTZ)	<b>0.00065</b>
Monotherapy GEN – (PTZ -> GEN)	<b>0.00065</b>
Monotherapy GEN – (PTZ + GEN)	<b>0.00065</b>
Monotherapy PTZ – (GEN -> PTZ)	0.07026
Monotherapy PTZ – (PTZ -> GEN)	0.54939
Monotherapy PTZ – (PTZ + GEN)	0.32107
(GEN -> PTZ) – (PTZ -> GEN)	<b>0.00870</b>
(GEN -> PTZ) – (PTZ + GEN)	<b>0.00713</b>
(PTZ-> GEN) – (PTZ + GEN)	0.54939

<sup>a</sup> Post hoc comparisons following a significant nested permutation ANOVA were carried out using Pairwise Wilcoxon Rank Sum Tests in R version 4.0.3. *P* values were adjusted for multiple comparisons using the False Discovery Rate (FDR) method: Significant *P* values are given in bold.

**Supplementary Table S6. ANOVA table for resistance (MIC) on GEN of evolved populations <sup>a</sup>**

Source of variation	Sums of squares	df	F-ratio	P
Meta-treatment	0.468	1	1.3794	0.252
Meta-treatment: Treatment	134.522	8	49.5211	< <b>0.0001</b>
Residuals	17.317	51		

<sup>a</sup> The effects of the two factors, Meta-treatment and Treatment on the response variable resistance (MIC) on GEN was assessed using a nested permutation ANOVA (Treatment nested in Meta-treatment). The analysis was performed in R version 4.0.3 using the model *Anova(aov(diffANC ~ Meta.treatment/Treatment, data = GENMICdf), type = 2)*. 10000 runs of permutation were carried out. Significant *P* values are indicated in bold.

**Supplementary Table S7. Post hoc tests comparing Treatments within a Meta-treatment for resistance (MIC) on GEN of evolved populations <sup>a</sup>**

Comparison	Adjusted <i>P</i>
<b>No mixing</b>	
Drug free – Monotherapy GEN	<b>0.0077</b>
Drug free – Monotherapy PTZ	<b>0.0123</b>
Drug free – (GEN -> PTZ)	<b>0.0097</b>
Drug free – (PTZ + GEN)	0.0853
Monotherapy GEN – Monotherapy PTZ	1.0000
Monotherapy GEN – (GEN -> PTZ)	0.0764
Monotherapy GEN – (PTZ + GEN)	0.0961
Monotherapy PTZ – (GEN -> PTZ)	0.2061
Monotherapy PTZ – (PTZ + GEN)	0.2061
(GEN -> PTZ) – (PTZ + GEN)	0.2059
<b>Mixing</b>	
Drug free – Monotherapy GEN	<b>0.0058</b>
Drug free – Monotherapy PTZ	0.5990
Drug free – (GEN -> PTZ)	<b>0.0058</b>
Drug free – (PTZ + GEN)	<b>0.0226</b>
Monotherapy GEN – Monotherapy PTZ	<b>0.0122</b>

Monotherapy GEN – (GEN -> PTZ)	<b>0.0058</b>
Monotherapy GEN – (PTZ + GEN)	<b>0.0259</b>
Monotherapy PTZ – (GEN -> PTZ)	<b>0.0058</b>
Monotherapy PTZ – (PTZ + GEN)	<b>0.0259</b>
(GEN -> PTZ) – (PTZ + GEN)	0.4218

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<sup>a</sup> Post hoc comparisons following a significant nested permutation ANOVA were carried out using Pairwise Wilcoxon Rank Sum Tests in R version 4.0.3. *P* values were adjusted for multiple comparisons using the False Discovery Rate (FDR) method: Significant *P* values are given in bold.

**Supplementary Table S8. ANOVA table for resistance (MIC) on PTZ of evolved populations <sup>a</sup>**

Source of variation	Sums of squares	<i>df</i>	<i>F-ratio</i>	<i>P</i>
<b>Meta-treatment</b>	98.570	1	139.589	<b>&lt; 0.0001</b>
<b>Meta-treatment: Treatment</b>	115.818	8	20.502	<b>&lt; 0.0001</b>
<b>Residuals</b>	36.013	51		

<sup>a</sup> The effects of the two factors, Meta-treatment and Treatment on the response variable resistance (MIC) on PTZ was assessed using a nested permutation ANOVA (Treatment nested in Meta-treatment). The analysis was performed in R version 4.0.3 using the model *Anova(aov(diffANC ~ Meta.treatment/Treatment, data = PITMICdf), type = 2)*. 10000 runs of permutation were carried out. Significant *P* values are indicated in bold.

**Supplementary Table S9. Post hoc tests comparing Treatments within a Meta-treatment for resistance (MIC) on PTZ of evolved populations <sup>a</sup>**

Comparison	<i>Adjusted P</i>
<b>No mixing</b>	
<b>Drug free – Monotherapy GEN</b>	0.96
<b>Drug free – Monotherapy PTZ</b>	0.72
<b>Drug free – (GEN -&gt; PTZ)</b>	0.40
<b>Drug free – (PTZ + GEN)</b>	0.28
<b>Monotherapy GEN – Monotherapy PTZ</b>	0.68
<b>Monotherapy GEN – (GEN -&gt; PTZ)</b>	0.28
<b>Monotherapy GEN – (PTZ + GEN)</b>	0.28
<b>Monotherapy PTZ – (GEN -&gt; PTZ)</b>	0.37
<b>Monotherapy PTZ – (PTZ + GEN)</b>	0.28
<b>(GEN -&gt; PTZ) – (PTZ + GEN)</b>	0.41
<b>Mixing</b>	
<b>Drug free – Monotherapy GEN</b>	<b>0.0255</b>
<b>Drug free – Monotherapy PTZ</b>	<b>0.0065</b>
<b>Drug free – (GEN -&gt; PTZ)</b>	<b>0.0059</b>
<b>Drug free – (PTZ + GEN)</b>	<b>0.0255</b>
<b>Monotherapy GEN – Monotherapy PTZ</b>	<b>0.0069</b>

<b>Monotherapy GEN – (GEN -&gt; PTZ)</b>	<b>0.0059</b>
<b>Monotherapy GEN – (PTZ + GEN)</b>	<b>0.0262</b>
Monotherapy PTZ – (GEN -> PTZ)	0.7814
Monotherapy PTZ – (PTZ + GEN)	0.0724
(GEN -> PTZ) – (PTZ + GEN)	0.1478

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<sup>a</sup> Post hoc comparisons following a significant nested permutation ANOVA were carried out using Pairwise Wilcoxon Rank Sum Tests in R version 4.0.3. *P* values were adjusted for multiple comparisons using the False Discovery Rate (FDR) method: Significant *P* values are given in bold.

**Supplementary Table S10. ANOVA table for resistance (AUC) on GEN of evolved populations <sup>a</sup>**

Source of variation	Sums of squares	df	F-ratio	P
Meta treatment	0.0000	1	0.0008	0.9793
Meta treatment: Treatment	8.4588	10	27.2600	< <b>0.0001</b>
Residuals	1.5515	50		

<sup>a</sup> The effects of the two factors, Meta-treatment and Treatment on the response variable resistance (AUC) on GEN was assessed using a nested permutation ANOVA (Treatment nested in Meta-treatment). The analysis was performed in R version 4.0.3 using the model *Anova(aov(GEN ~ Meta.treatment/Treatment, data = AUCdf), type = 2)*. 10000 runs of permutation were carried out. Significant *P* values are indicated in bold.

**Supplementary Table S11. Post hoc tests comparing Treatments within a Meta-treatment for resistance (AUC) on GEN of evolved populations <sup>a</sup>**

Comparison	Adjusted P
<b>No mixing</b>	
Drug free – Monotherapy GEN	<b>0.0087</b>
Drug free – Monotherapy PTZ	<b>0.0625</b>
Drug free – (GEN -> PTZ)	<b>0.0087</b>
Drug free – (PTZ -> GEN)	<b>0.0303</b>
Drug free – (PTZ + GEN)	0.5510
Monotherapy GEN – Monotherapy PTZ	0.9740
Monotherapy GEN – (GEN -> PTZ)	1.0000
Monotherapy GEN – (PTZ -> GEN)	0.5510
Monotherapy GEN – (PTZ + GEN)	0.9890
Monotherapy PTZ – (GEN -> PTZ)	1.0000
Monotherapy PTZ – (PTZ -> GEN)	0.6667
Monotherapy PTZ – (PTZ + GEN)	0.7500
(GEN -> PTZ) – (PTZ -> GEN)	0.5510
(GEN -> PTZ) – (PTZ + GEN)	0.9890
(PTZ-> GEN) – (PTZ + GEN)	0.6667



<b>Mixing</b>	
Drug free – Monotherapy GEN	<b>0.0058</b>
Drug free – Monotherapy PTZ	<b>0.0350</b>
Drug free – (GEN -> PTZ)	<b>0.0023</b>
Drug free – (PTZ -> GEN)	<b>0.0260</b>
Drug free – (PTZ + GEN)	<b>0.0050</b>
Monotherapy GEN – Monotherapy PTZ	<b>0.0198</b>
Monotherapy GEN – (GEN -> PTZ)	0.5611
Monotherapy GEN – (PTZ -> GEN)	0.1786
Monotherapy GEN – (PTZ + GEN)	0.1712
Monotherapy PTZ – (GEN -> PTZ)	<b>0.0058</b>
Monotherapy PTZ – (PTZ -> GEN)	0.0595
Monotherapy PTZ – (PTZ + GEN)	<b>0.0130</b>
(GEN -> PTZ) – (PTZ -> GEN)	0.0727
(GEN -> PTZ) – (PTZ + GEN)	0.2636
(PTZ-> GEN) – (PTZ + GEN)	1.0000

<sup>a</sup> Post hoc comparisons following a significant nested permutation ANOVA were carried out using Pairwise Wilcoxon Rank Sum Tests in R version 4.0.3. *P* values were adjusted for multiple comparisons using the False Discovery Rate (FDR) method: Significant *P* values are given in bold.

**Supplementary Table S12. ANOVA table for resistance (AUC) on PTZ of evolved populations <sup>a</sup>**

Source of variation	Sums of squares	df	F-ratio	P
Meta treatment	3.3197	1	75.569	< <b>0.0001</b>
Meta treatment: Treatment	3.6647	10	8.342	< <b>0.0001</b>
Residuals	2.1965	50		

<sup>a</sup> The effects of the two factors, Meta-treatment and Treatment on the response variable resistance (AUC) on PTZ was assessed using a nested permutation ANOVA (Treatment nested in Meta-treatment). The analysis was performed in R version 4.0.3 using the model *Anova(aov(PTZ ~ Meta.treatment/Treatment, data = AUCdf), type = 2)*. 10000 runs of permutation were carried out. Significant *P* values are indicated in bold.

**Supplementary Table S13. Post hoc tests comparing Treatments within a Meta-treatment for resistance (AUC) on PTZ of evolved populations <sup>a</sup>**

Comparison	Adjusted P
<b>No mixing</b>	
Drug free – Monotherapy GEN	0.083
Drug free – Monotherapy PTZ	0.083
Drug free – (GEN -> PTZ)	0.677
Drug free – (PTZ -> GEN)	0.083
Drug free – (PTZ + GEN)	0.750
Monotherapy GEN – Monotherapy PTZ	1.000
Monotherapy GEN – (GEN -> PTZ)	0.985
Monotherapy GEN – (PTZ -> GEN)	1.000
Monotherapy GEN – (PTZ + GEN)	1.000
Monotherapy PTZ – (GEN -> PTZ)	1.000
Monotherapy PTZ – (PTZ -> GEN)	1.000
Monotherapy PTZ – (PTZ + GEN)	1.000
(GEN -> PTZ) – (PTZ -> GEN)	1.000
(GEN -> PTZ) – (PTZ + GEN)	1.000
(PTZ-> GEN) – (PTZ + GEN)	1.000

<b>Mixing</b>	
Drug free – Monotherapy GEN	<b>0.0078</b>
Drug free – Monotherapy PTZ	0.1088
Drug free – (GEN -> PTZ)	0.1573
Drug free – (PTZ -> GEN)	0.8312
Drug free – (PTZ + GEN)	0.5170
Monotherapy GEN – Monotherapy PTZ	<b>0.0198</b>
Monotherapy GEN – (GEN -> PTZ)	<b>0.0078</b>
Monotherapy GEN – (PTZ -> GEN)	0.1948
Monotherapy GEN – (PTZ + GEN)	<b>0.0130</b>
Monotherapy PTZ – (GEN -> PTZ)	0.6043
Monotherapy PTZ – (PTZ -> GEN)	0.0670
Monotherapy PTZ – (PTZ + GEN)	<b>0.0130</b>
(GEN -> PTZ) – (PTZ -> GEN)	<b>0.0260</b>
(GEN -> PTZ) – (PTZ + GEN)	<b>0.0078</b>
(PTZ-> GEN) – (PTZ + GEN)	0.9048

<sup>a</sup> Post hoc comparisons following a significant nested permutation ANOVA were carried out using Pairwise Wilcoxon Rank Sum Tests in R version 4.0.3. *P* values were adjusted for multiple comparisons using the False Discovery Rate (FDR) method: Significant *P* values are given in bold.

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### GENE SHARING AMONG PLASMIDS AND CHROMOSOMES REVEALS BARRIERS FOR ANTIBIOTIC RESISTANCE GENE TRANSFER

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The emergence of antibiotic resistant bacteria is a major threat to modern medicine. Rapid adaptation to antibiotics is often mediated by the acquisition of plasmids carrying antibiotic resistance (ABR) genes. Nonetheless, the determinants of plasmid-mediated ABR gene transfer remain debated. Here we show that the propensity of ABR gene transfer via plasmids is higher for accessory chromosomal ABR genes in comparison to core chromosomal ABR genes, regardless of the resistance mechanism. Analyzing the pattern of ABR gene occurrence in the genomes of 2,635 *Enterobacteriaceae* isolates we find that 33% of the 416 ABR genes are shared between chromosomes and plasmids. Phylogenetic reconstruction of ABR genes occurring on both plasmids and chromosomes supports their evolution by lateral gene transfer. Furthermore, accessory ABR genes (encoded in  $\leq 10\%$  of the chromosomes) occur more abundantly in plasmids in comparison to core ABR genes (encoded in  $\geq 90\%$  of the chromosomes). The pattern of ABR gene occurrence in plasmids and chromosomes is similar to the total *Escherichia* genome. Our results thus indicate that the previously recognized barriers for gene acquisition by lateral gene transfer apply also to ABR genes. We propose that the functional complexity of the underlying ABR mechanism is an important determinant of ABR gene transferability.

## Introduction

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Bacteria harbour vast potential to rapidly adapt to different selective environments, including environments defined by antibiotics. Notably, antibiotic resistance (ABR) predates the clinical use of antibiotics: ABR genes have been reported from permafrost [1] and ancient human remains [2]. Today, the ability of bacteria to adapt to antibiotics forms the core of the antibiotic resistance crisis [3]. Drug therapy is aimed to kill bacteria by targeting key components and processes that are crucial for their reproduction; the repertoire of genetic variants giving rise to antibiotic resistance therefore includes many core genes [4]. For example, bacterial efflux pumps, which function in resistance to diverse antibiotics, have multiple roles in bacterial physiology in addition to removal of antibiotics from the cell, including the extrusion of heavy metals and cell signalling molecules [5]. Indeed, genes encoding efflux pumps and their regulation are typically part of bacterial core genomes, i.e., they are nearly universally present in bacterial genomes [6]. By contrast, other resistance mechanisms are encoded by genes in the accessory genome and can be found also on mobile genetic elements (e.g., genes involved in target replacement or target protection) [7]. While the emergence of antibiotic resistance under long-term drug therapy is common via *de novo* mutations, lateral transfer of ABR genes by mobile genetic elements has the potential to disseminate resistance to various antibiotics within microbial communities in clinical settings, agriculture and also the environment [8,9].

The acquisition of ABR genes can be mediated by three main transfer mechanisms: transformation, transduction, and conjugation. Bacterial organisms that are naturally competent may acquire ABR genes via natural transformation of mobile genetic elements carrying ABR genes, including transposons, IS elements and integrons (e.g., [10]). Transduction can cause the transfer of ABR genes encoded in the genome of lysogenic phages (e.g., [11,12]) or the transfer of ABR gene-containing plasmids by phages (e.g., [13]). Notwithstanding, plasmid-mediated ABR gene transfer occurs most commonly via conjugation, which facilitates the transfer of conjugative and mobilizable plasmids (e.g., [14]). The dissemination of ABR genes by plasmids is often associated with the evolution of plasmid-encoded integrons that may comprise multiple ABR genes [15]. The type of vehicle that mediates the ABR gene transfer may have implications for the phylogenetic range of the transfer event. Phage-mediated gene transfer is often restricted to closely related donors and recipients due to phage host specificity [12]. Similarly, the apparent taxonomic pattern in networks of plasmid sequence similarity [16] suggests that plasmid host range constrains the range of plasmid-mediated gene transfer to closely related donor and recipients (with the exception of broad host range plasmids, e.g., [17]).

The prevalence of gene transfer may furthermore depend on genetic and functional characteristics of the transferred gene. Genes that encode for proteins whose function depends on interaction with multiple proteins, e.g., within the same complex or cellular process, are rarely transferred likely because their acquisition leads to interference with the component sociometry [18,19]. An increase in gene copy number following lateral gene transfer may often lead to disadvantageous dose effect, which has been shown to pose a barrier to lateral gene transfer [20,21]. The acquisition of novel laterally transferred genes may have deleterious effects on the recipient due to diverse reasons ranging from the consequences of foreign DNA

integration into the genome to proper expression and function of the protein product in the cell (e.g., [22]; reviewed in [23]). A potential disruption of chromosomal architecture may be minimized if genes are transferred by and expressed from extra-chromosomal genetic elements like plasmids (i.e., assuming non-integrative plasmids). Nonetheless, ABR gene expression from a plasmid locus may be accompanied by fitness cost to the host due to various other reasons. For example, ABR mechanisms that rely on drug inactivation may require a lower energetic investment of the cell in comparison to efflux pumps (i.e., in ATP) [24]. Such trade-offs in the adaptation of ABR plasmids in newly colonized hosts are expected to have consequence for the evolution of plasmid ABR gene content.

Plasmid gene content is furthermore expected to be subject to purifying selection due to genetic conflicts between plasmid and chromosomally encoded loci that affect the same phenotype [25]. Indeed, a recent study suggests that the evolution of plasmid ABR gene content depends on the level of genetic conflicts with chromosomally encoded genes, which is strongest for ABR genes encoding efflux pumps and weakest for ABR genes encoding antibiotic target inactivation mechanisms [7]. Considering the association between ABR gene classification into core or accessory genome and the ABR mechanism, we hypothesize that the prevalence of ABR genes on plasmids depends on the ABR gene occurrence in bacterial chromosomes. Here we examine ABR gene occurrence in bacterial plasmids and chromosomes using a large-scale analysis of three *Enterobacteriaceae* genera: *Escherichia*, *Salmonella*, and *Klebsiella*, for which a large number of genomes is publicly available. We further assessed whether ABR gene occurrence on plasmids and chromosomes is associated with the resistance mechanism and inferred ABR gene transfer events using phylogenetics and the temporal pattern of gene occurrence in plasmids and chromosomes.

## Data and Methods

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To study the pattern of ABR gene occurrence in bacterial replicons (Chromosomes and plasmids) we examined all complete genomes of plasmid-carrying isolates in the genera *Escherichia*, *Salmonella* and *Klebsiella* from the NCBI genome database (electronic supplementary material, table S1). Assembly and annotation files of 2,580 genomes were downloaded from NCBI RefSeq database [26] (version 01/2021). Additional 78 complete genomes lacking RefSeq annotation were downloaded from GenBank database. Protein-coding genes of the 78 genomes were annotated using Prokka [27] (version 1.14.6, with parameter `--kingdom Bacteria --gcode 11 --genus {Escherichia/ Salmonella/ Klebsiella} --usegenus --evaluate 1e-9`). Isolate metadata was downloaded from BioSample database. Samples lacking host information were examined in detail. A total of 23 strains sequenced within the framework of laboratory experiments were excluded. The surveyed genomes include: 1,169 chromosomes and 3,286 plasmids from *Escherichia*; 846 chromosomes and 3,020 plasmids from *Klebsiella*; 620 chromosomes and 1,090 plasmids from *Salmonella*. Homologs to genes that may provide ABR were identified based on the comprehensive antibiotic resistance database (CARD; version 3.1.0) using the Resistance Gene Identifier tool (RGI; version 5.1.1, with parameter `--clean`) [28]. The application of RGI uncovered 126,910 protein-coding genes; of these, we excluded 4,774 protein-coding genes whose sequence has less than 70% identity and sequence was <90% shorter and >30% longer in comparison to the CARD ABR genes. Of the

total 3,044 genes in CARD, 416 (14%) ABR genes had 122,136 homologs in the examined isolates. Of those, we further excluded 9 genes classified into multiple antibiotic resistance mechanism classes.

The inference of protein families in *Escherichia* was performed using a dataset of 599 completely sequenced *Escherichia* strains that were download from NCBI RefSeq database (version 2018) (previously reported in [29]). Of the total strains, 416 plasmid carrying isolates were retained for further analysis (electronic supplementary material, table S2). Genome-wise reciprocal best hits (RBHs) of protein sequences were identified using MMseqs2 [30] (version 13.45111, with module easy-rbh applying a threshold of E-value  $\leq 1e-10$ ). RBHs were further compared by global alignment using parasail-python [31] (version 1.2.4, with the Needleman-Wunsch algorithm). Sequence pairs with  $\geq 30\%$  identical amino acids were clustered into protein families using the Markov clustering algorithm (MCL) [32] (version 12-135, with parameter `-abc -I 2.0`).

Data analysis, statistical inference and visualization was performed with R (version 4.0) and MATLAB (version 2018b). Testing for a bias in ABR gene occurrence towards plasmids or chromosomes was performed by comparing the frequency of ABR gene occurrence on both replicons to the frequency of the remaining genes on both replicons (using Fisher's exact test with `fisher.test` function in R).

For the phylogenetic reconstruction, non-redundant protein sequences of selected ABR gene were aligned using MAFFT [33] (version v7.475). Due to the large number of identical protein sequences among the ABR gene homologs, the sequences were filtered to include non-redundant amino acid sequences at the resolution of replicon and genera. Phylogenetic trees were reconstructed using IQ-Tree [34] (version 1.6.12) with restricted automatic model selection to the Le and Gascuel (LG) Model [35] and the LG4X Model [36] as additional alternative (with parameter `-mset LG -madd LG4X`). The LG model was chosen due to its suitability for the inference of phylogenetic trees using protein sequences from bacterial organisms using maximum likelihood methods. The resulting trees were rooted using the midpoint criterion and visualized using iTOL [37] v6 (<https://itol.embl.de/>).

## Results

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### Plasmid ABR gene content is associated with gene prevalence on chromosomes

The survey for genes that putatively confer ABR revealed 2,810 (38%) plasmids that encode an ABR gene homolog (termed here ABR plasmids). The ABR plasmids typically encode multiple ABR genes with the median ABR genes per plasmid ranging between four genes in *Escherichia* and six genes in *Salmonella*. The proportion of ABR plasmids was similar among the three genera: 34% (1,131) in *Escherichia*, 42% (1,256) in *Klebsiella*, and 39% (423) in *Salmonella*. The genomes of all isolates in the three genera had at least one homolog to an ABR gene encoded in the chromosome.

The occurrence of ABR gene homologs in *Escherichia* plasmids and chromosomes reveals three classes: (1) ABR genes exclusively found on plasmids, (2) ABR genes found exclusively on chromosomes, and (3) ABR genes found on both plasmids and chromosomes

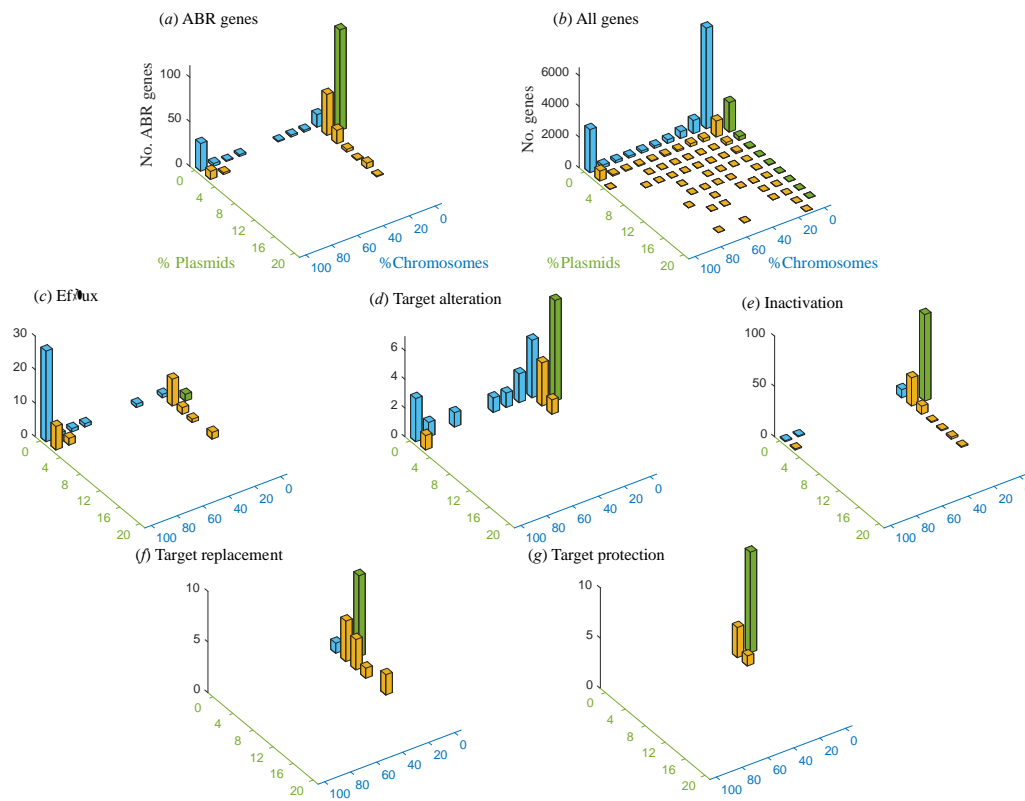


(figure 1a). The pattern of gene occurrence on plasmids and chromosomes further reveals two main groups: ABR genes encoded on plasmids and shared with a small proportion of the chromosomes ( $\leq 10\%$ ) – these genes are considered to be part of the accessory genome, and ABR genes that are encoded on plasmids and are shared with  $\geq 90\%$  of the chromosomes – these genes are considered to be part of the core genome (see yellow bars in figure 1a). A similar pattern of ABR gene occurrence on plasmids and chromosomes is observed in the genomes of *Klebsiella* and *Salmonella* isolates (electronic supplementary material, figures S1 and S2). To compare the pattern of ABR gene occurrence to the total *Escherichia* genes, we repeated the analysis using a dataset of protein families in *Escherichia* isolates. The results show that the ABR gene occurrence in plasmids and chromosomes bears similarity to the distribution of the total gene families in *Escherichia* genomes (figure 1b): in both datasets homologs of core chromosomal genes found in  $\geq 90\%$  of the chromosomes are less abundant in plasmids in comparison to homologs of accessory chromosomal genes found in  $\leq 10\%$  of the chromosomes. The similar pattern of shared genes among plasmids and chromosomes observed for the ABR genes and the total genome indicates that the association between ABR gene prevalence in plasmids and chromosomes is general within *Escherichia* genomes rather than a unique property of ABR genes.

Previous studies suggested that the occurrence of ABR genes in plasmids may be related to the underlying mechanism of ABR [7,24]. Indeed, further examination of the ABR gene occurrence on plasmids and chromosomes reveals differences across the ABR mechanism classes. ABR genes that are involved in the expression of efflux pumps are typically core chromosomal genes that are rarely found on plasmids (figure 1c). By contrast, ABR genes classified as antibiotic target alteration include both chromosome- and plasmid- exclusive genes, with rare gene sharing between plasmids and chromosomes (figure 1d). ABR genes classified as antibiotic inactivation comprise mostly plasmid-specific genes with some genes occurring in chromosomes as accessory genes and rarely as core genes (figure 1e). Genes classified as target protection and target replacement are encoded on a small number of plasmids and chromosomes (figures 1f and 1g).

To further examine the mode of ABR gene evolution, that is, if it is mainly by vertical inheritance or lateral gene transfer, we explored biases in the prevalence of ABR gene homologs towards plasmids and chromosomes (termed hereafter ‘replicon type’) for each of the ABR genes in our dataset. The results of the statistical analysis confirm that most of the ABR genes encoding for efflux pumps are significantly more frequent on chromosomes (table 1A, electronic supplementary material, table S3). Nonetheless, some exceptional efflux-coding genes are enriched on plasmids, including *tetA/B*, *floR*, and *qacED1*, whose homologs are mainly encoded in plasmids in all three genera (electronic supplementary material, table S3). The distribution of ABR genes that encode for antibiotic target alteration proteins is balanced between chromosomes and plasmids in *Escherichia*, while in *Klebsiella* and *Salmonella* it is biased towards plasmids. Examples for chromosomal antibiotic target alteration genes encode the translation elongation factor EF-Tu, that is found as an exclusive core gene in all genera, and *bacA*, an undecaprenyl pyrophosphate phosphatase [38], that is a core chromosomal gene in *Escherichia* and *Salmonella*. Examples for genes encoding antibiotic target alteration

proteins that are overrepresented on plasmids include *ermB* and *mcr-1* (electronic supplementary material, table S3).



**Figure 1.** A comparison of gene prevalence in plasmids and chromosomes in *Escherichia* isolates. Plots in the figure present 2-dimensional histograms of gene occurrence in plasmids (green axis) and chromosomes (blue axis). The height of bars in the histogram corresponds to the number of genes that are encoded in the same frequency group on chromosomes and plasmids. The bar coordinates correspond to the proportion of replicons (plasmids or chromosomes) where the genes occur. Note that no gene occurred in >20% of the plasmids; genes occurring in 100% of the chromosomes correspond to core gene families. Genes occurring only on chromosomes are shown with blue bars. Genes occurring only on plasmids are shown with green bars. Yellow bars correspond to genes that occur in both plasmids and chromosomes. **(a)** ABR genes in the examined isolates (data supplied in electronic supplementary material, table S3). **(b)** Gene occurrence of all *Escherichia* gene families. **(c-g)** ABR gene according to their classification into ABR mechanism (as in CARD [28]).

ABR genes in the inactivation class are typically encoded on plasmids with rare genes showing a bias towards the chromosome (table 1A). Those exceptional genes include *ampH*, which is a core chromosomal gene in all three genera. This gene encodes for a penicillin binding protein that has a function in the determination of cell shape in *E. coli* and can confer resistance to  $\beta$ -lactam antibiotics [39]. Another example is *ampC1* which encodes for a  $\beta$ -lactamase and is nearly universally encoded in *Escherichia* and *Salmonella* but absent in *Klebsiella* genomes. Additional examples are *aac(6')-Iy* and *aac(6')-Iaa*, both encode the function of aminoglycoside acetyltransferases, which are exclusively found in *Salmonella* chromosomes. The genes *fosA5* and *fosA6*, mediating resistance against Fosfomycin, have been reported in *Escherichia* [40], yet in our extensive dataset they are observed exclusively as accessory chromosomal genes in *Klebsiella* (electronic supplementary material, table S3).

**Table 1.** Comparison of ABR frequency in plasmid and chromosomes. **(a)** The number of ABR genes having a bias towards plasmid or chromosome according to the resistance mechanism classification (using Fisher's exact test in contingency analysis,  $\alpha=0.05$  and correction for multiple comparisons with FDR [78]). No bias to the chromosome was found in ABR genes for antibiotics target protection and replacement (marked with a "-"). **(b)** ABR genes whose relative frequency in plasmid and chromosomes differs among the three tested genera. The table shows ABR gene occurrence on plasmids and chromosomes in the three genera. The ABR gene frequency on both replicons was compared with Fisher's exact test (the P-value is reported in addition to adjusted P-value for multiple comparisons with FDR). Chromosome- and plasmid-exclusive genes and genes absent in any of the genera were excluded from this analysis (a total of 98 ABR genes were tested). Genes discussed in the result sections c-e are highlighted in bold.

(a)

Genus	antibiotic efflux		antibiotic inactivation		antibiotic target alteration		antibiotic target protection		antibiotic target replacement	
	Chromosome	Plasmid	Chromosome	Plasmid	Chromosome	Plasmid	Chromosome	Plasmid	Chromosome	Plasmid
<i>Escherichia</i>	40(74%)	14(26%)	3(2%)	126(98%)	10(50%)	10(50%)	-	13(100%)	-	17(100%)
<i>Klebsiella</i>	14(50%)	14(50%)	3(3%)	100(97%)	8(40%)	12(60%)	-	11(100%)	-	13(100%)
<i>Salmonella</i>	20(61%)	13(39%)	4(5%)	74(95%)	5(38%)	8(62%)	-	9(100%)	-	13(100%)

(b)

ARO	Gene	Resistance.Mechanism	Escherichia		Salmonella		Klebsiella		p	p.adj
			Chromosome	Plasmid	Chromosome	Plasmid	Chromosome	Plasmid		
3003922	<b><i>oqxA</i></b>	antibiotic efflux	2	45	2	36	728	1	1.02e-105	1.00e-103
3003923	<b><i>oqxB</i></b>	antibiotic efflux	2	31	2	19	162	0	5.51e-45	2.70e-43
3000165	<b><i>tetA</i></b>	antibiotic efflux	62	283	20	165	13	232	1.02e-05	7.71e-05
3000166	<b><i>tetB</i></b>	antibiotic efflux	69	100	0	0	0	0	-	-
3005010	<b><i>qacED1</i></b>	antibiotic efflux	55	291	68	137	116	371	1.73e-05	1.21e-04
3000873	<b><i>bla</i><sub>TEM-1</sub></b>	antibiotic inactivation	65	333	53	133	9	404	6.65e-22	2.17e-20
3004290	<b><i>ampC</i></b>	antibiotic inactivation	1119	1	2	0	0	0	-	-
3003689	<b><i>mcr-1</i></b>	antibiotic target alteration	0	0	0	26	0	8	-	-
3002790	<b><i>qnrS1</i></b>	antibiotic target protection	8	105	2	6	2	132	6.61e-02	2.06e-01
3000412	<b><i>sul2</i></b>	antibiotic target replacement	90	287	55	187	21	272	2.00e-09	2.79e-08
3002705	<b><i>floR</i></b>	antibiotic efflux	33	143	33	121	2	63	9.17e-04	4.99e-03
3000168	<b><i>tetD</i></b>	antibiotic efflux	6	1	1	4	2	50	3.69e-06	3.28e-05
3002660	<b><i>APH(6)-Id</i></b>	antibiotic inactivation	82	270	60	173	17	249	1.70e-10	4.16e-09
3002639	<b><i>APH(3'')-Ib</i></b>	antibiotic inactivation	82	263	59	167	18	245	4.26e-10	8.34e-09
3001059	<b><i>bla</i><sub>SHV-1</sub></b>	antibiotic inactivation	0	2	0	1	144	7	2.01e-04	1.23e-03
3002601	<b><i>aadA</i></b>	antibiotic inactivation	43	130	10	76	3	133	8.62e-09	1.06e-07
3001877	<b><i>bla</i><sub>CTX-M-14</sub></b>	antibiotic inactivation	23	43	2	13	7	51	7.64e-03	3.57e-02
3002013	<b><i>CMY-2</i></b>	antibiotic inactivation	22	42	6	70	0	7	1.81e-04	1.18e-03
3002539	<b><i>AAC(3)-IV</i></b>	antibiotic inactivation	5	37	4	45	12	21	4.48e-03	2.20e-02
3002603	<b><i>aadA3</i></b>	antibiotic inactivation	5	29	27	29	97	44	1.70e-08	1.85e-07
3002683	<b><i>catI</i></b>	antibiotic inactivation	35	27	26	35	9	81	5.23e-10	8.54e-09
3001926	<b><i>bla</i><sub>CTX-M-65</sub></b>	antibiotic inactivation	4	13	1	20	0	76	5.21e-04	3.00e-03
3004656	<b><i>catII</i></b>	antibiotic inactivation	0	7	4	19	1	88	8.84e-03	3.94e-02
3002240	<b><i>bla</i><sub>CARB-1</sub></b>	antibiotic inactivation	0	4	25	0	0	2	1.36e-06	1.33e-05
3001397	<b><i>bla</i><sub>OXA-2</sub></b>	antibiotic inactivation	3	0	0	1	0	10	2.75e-03	1.42e-02
3000410	<b><i>sul1</i></b>	antibiotic target replacement	53	286	66	140	76	371	8.05e-06	6.58e-05
3002862	<b><i>dfrA7</i></b>	antibiotic target replacement	13	2	21	23	0	1	9.83e-03	4.19e-02

Purifying selection on plasmid gene content due to genetic conflicts is expected to differ among taxa having different composition of their core genome. Nonetheless, the bias of ABR genes towards specific replicon type (i.e., plasmids or chromosomes) is generally similar among the three tested genera in our dataset, likely since they are closely related and their core ABR genome largely overlaps. Nonetheless, 23 ABR genes stand out as exception (table 1b). Those are mostly plasmid genes with variable presence on the chromosomes, i.e., they can be considered accessory genes. One extreme case is the antibiotic efflux gene *oqx*A, which is a core chromosomal gene in *Klebsiella* and enriched on plasmids in the other two genera. Among the exceptional genes encoding antibiotic inactivation proteins, the gene *bla*<sub>CARB-1</sub> is found as an accessory chromosomal gene in *Salmonella* with no bias towards specific replicon type but only on plasmids in *Escherichia* and *Klebsiella* (electronic supplementary material, table S2).

### **ABR gene transfer between plasmids and chromosomes**

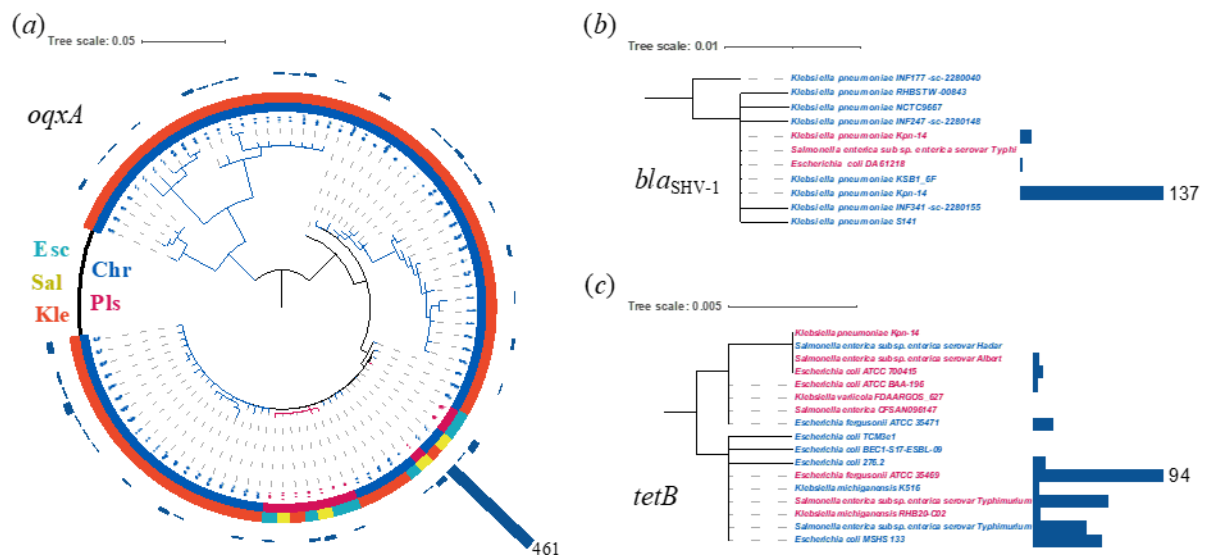
Our results reveal 133 (33%) ABR genes that are found on both replicons in at least one genus thus indicating that they have been laterally transferred. Of those, 75 ABR genes were observed on both plasmid and chromosome within the same isolate genome in at least one strain. The majority of genes that we identified at least once as duplicated, are enriched on plasmids (65 out of 75 genes); of those, 45 genes encode proteins that function in antibiotic inactivation (electronic supplementary material, table S4). Only five of the 75 duplicated genes are enriched for a chromosome location; these correspond to four genes encoding efflux pumps and the translation elongation factor (target alteration). The presence of duplicated ABR genes on both replicons in the same isolate is relatively rare. We note here that deleterious effects in the context of gene gain following lateral gene transfer [23] are expected not only for the recipient, but rather also for the donor, following gene transfer from chromosomes to plasmids. Taken together, our results suggest that genes that are duplicated on the plasmid and chromosome in the same isolate often correspond to genes introduced into the genus as plasmid genes.

To further study the evolutionary history of genes shared between plasmids and chromosomes we reconstructed the phylogenetic trees of the ABR genes and examined their topology. In what follows we present the results of 39 selected ABR genes exemplifying main trends in the data. The topology of ABR genes across replicon types and genera in the inferred phylogenetic trees confirm the occurrence of lateral gene transfer among genera and also replicon types in the history of all examined genes (electronic supplementary material, table S5). In the following, we present several prime examples.

### **Lateral gene transfer of genes encoding efflux pumps**

The genes *oqx*A and *oqx*B belong to the resistance-nodulation-cell division (RND) family of bacterial efflux pumps [41] and were first isolated from bacteria in swine manure from a farm where olaquinodox was used as a feed additive [42]. The proteins OqxA and OqxB, along with TolC, form an efflux pump that provides resistance against olaquinodox, chloramphenicol, quinolones, trimethoprim, tigecycline, nitrofurantoin, and also several detergents and disinfectants by reducing their concentrations in the cell [41,43]. Both *oqx*A and *oqx*B are almost strictly chromosomal genes in *Klebsiella* with *oqx*A occurring in 86% and *oqx*B occurring in 19% *Klebsiella* isolates. We identified a single *Klebsiella* isolate where *oqx*A

is present on both chromosome and plasmid (electronic supplementary material, table S4). The two genes have homologs in *Escherichia* and *Salmonella* plasmids. The phylogenetic trees of both genes indicate lateral transfer between *Klebsiella* chromosomes and plasmids of the other two genera (figure 2a; electronic supplementary material, figure S3a). We note that the *oqxA* protein sequences were nearly identical hence the phylogenetic reconstruction does not allow for a reliable inference of donors and recipients in the transfer event. To further study the history of the ABR genes we collected information on the isolation date of strains included in our analysis. The gene *oqxA* was documented early on in the 1950s as a chromosomal gene in *Klebsiella* (figure 3). The first documentation of *oqxA* on plasmid in our dataset is from 2004 in *Escherichia*; in recent years this gene has been found on both plasmids and chromosomes in all three genera. The gene *oqxB* was first documented in 2004 in *Klebsiella* chromosomes and *Escherichia* plasmids, while in recent years it has been described for both replicon types in all three genera (figure 3). The example of *oqxA* suggests that barriers to gene transfer of core chromosomal genes may differ between genera. The first report on plasmid-encoded *oqxAB* in *E. coli* in the literature dates back to the late 1990s [44]; today, after more than 20 years, that gene is still very rare in *Escherichia* and *Salmonella*. It is tenable to speculate that barriers to plasmid-mediated *oqxA* acquisition are present also in *Escherichia* and *Salmonella*.



**Figure 2.** Lateral gene transfer in the evolution of ABR genes. ABR gene name is written next to the phylogenetic tree. Bars next to the operational taxonomic units show the frequency of redundant amino acid sequences (with highest frequency noted). (a) The outer ring shows the genus of ABR gene origin, the inner ring shows whether the gene is encoded on a plasmid or chromosome. (b, c) Isolate label is coloured according to replicon type (plasmid genes in pink and chromosomal genes in blue). Most phylogenetic trees we reconstructed here for the ABR genes included polytomies, hence donor and recipients in the gene transfer events cannot be reliably inferred. Nonetheless, considering the presence of the examined ABR genes on both plasmids and chromosomes supports that they have been transferred at least once in their evolutionary history.

The Tet efflux pumps were the first efflux systems discovered in bacteria [45,46]. These major facilitator (MF) family exporters are membrane-associated proteins that pump out tetracycline from the cell in an energy-dependent manner [47]. Unlike the OqxAB-TolC system, the Tet efflux pumps provide resistance only against tetracyclines. Since the original report of *tetA*, many more *tet* genes have been discovered, a vast majority in Gram negative bacteria [48]. For our analysis we focused on the *tetA* and *tetB* genes, since both genes are enriched on plasmids and rarely found on chromosomes (<6% in all genera). The phylogenetic reconstruction shows that the protein sequence of *tetA* is more diversified in comparison to that of *tetB*, and that for both genes, multiple transfer events occurred between chromosomes and plasmids, and also across bacterial genera (figure 2c; electronic supplementary material, figure S3d). Furthermore, we identified 13 isolates (from all genera) where *tetA* is encoded in both replicon types and four *Escherichia* isolates that encode *tetB* on both replicon types. The gene *tetA* in our data was documented firstly on a plasmid in *Salmonella* in 1974, while *tetB* was identified on a plasmid in *Escherichia* at the same time (figure 3). In recent years, both genes have been found on both replicon types and in all three genera, yet *tetB* is seldomly documented in *Klebsiella*.

Another efflux gene that is mainly found on plasmids is *qacEΔ1*, a truncated variant of the *qacE* gene [49]. The *qac* genes are antiseptic efflux systems primarily exporting quaternary ammonium compounds. Their substrate range, however, is quite large and includes intercalating dyes, biguanidines, diamidines, and xanthenes. The gene *qacEΔ1* is frequently found in ABR gene cassettes isolated from bacteria exposed to these compounds compared to those which are not [50]. In our dataset, we find that *qacEΔ1* is very often encoded on both plasmids and chromosomes in the same isolate (71 *Klebsiella*, 11 *Escherichia* and 7 *Salmonella* isolates). The protein sequence is highly conserved between replicons and across all three genera. In our dataset, *qacEΔ1* was documented firstly on a plasmid in *Escherichia* in 1969, and then a *Klebsiella* plasmid in 1981; the first *qacEΔ1* on chromosome was identified in *Escherichia* in 1990 (figure 3).

### **Lateral gene transfer of genes encoding antibiotic inactivation proteins**

A prime example of antibiotic inactivation enzymes are the  $\beta$ -lactamases. These enzymes confer resistance to  $\beta$ -lactam antibiotics by cleaving their central  $\beta$ -lactam ring [51]. The gene *bla*<sub>TEM-1</sub> is the first of the TEM  $\beta$ -lactamases which are among the best-studied antibiotic resistance enzymes [52,53]. Although the original *bla*<sub>TEM-1</sub> gene confers resistance to penicillins and early cephalosporins, its later variants also provide resistance against second-, third- and fourth-generation cephalosporins, monobactams and  $\beta$ -lactamase inhibitors [53]. The *bla*<sub>TEM-1</sub> gene is mainly found on *Klebsiella* and *Escherichia* plasmids. The phylogenetic reconstruction indicates multiple transfer events and a high protein sequence conservation of *bla*<sub>TEM-1</sub> between replicons and across genera (electronic supplementary material, figure S3c). It was documented firstly on plasmid in both *Escherichia* and *Salmonella* in 1974, and first reported on chromosomes in *Salmonella* in 1981 (figure 3).

The AmpC  $\beta$ -lactamase of *Escherichia coli* was the first reported bacterial enzyme capable of degrading penicillin [54]. Although active against penicillins, this inducible enzyme has higher activity against cephalosporins and is an important determinant of resistance against

cephalosporins in the clinic [55]. The *ampC* gene in *Escherichia* is nearly exclusively a chromosomal gene. Additionally, homologs of *ampC* were found on a plasmid in one *Escherichia* isolate and the chromosome of two *Salmonella* isolates. The phylogenetic reconstruction indicates that the *Salmonella* homologs were acquired in two independent transfer events (electronic supplementary material, figure S3h).

### **Lateral transfer of ABR genes involved in target alteration, replacement and protection**

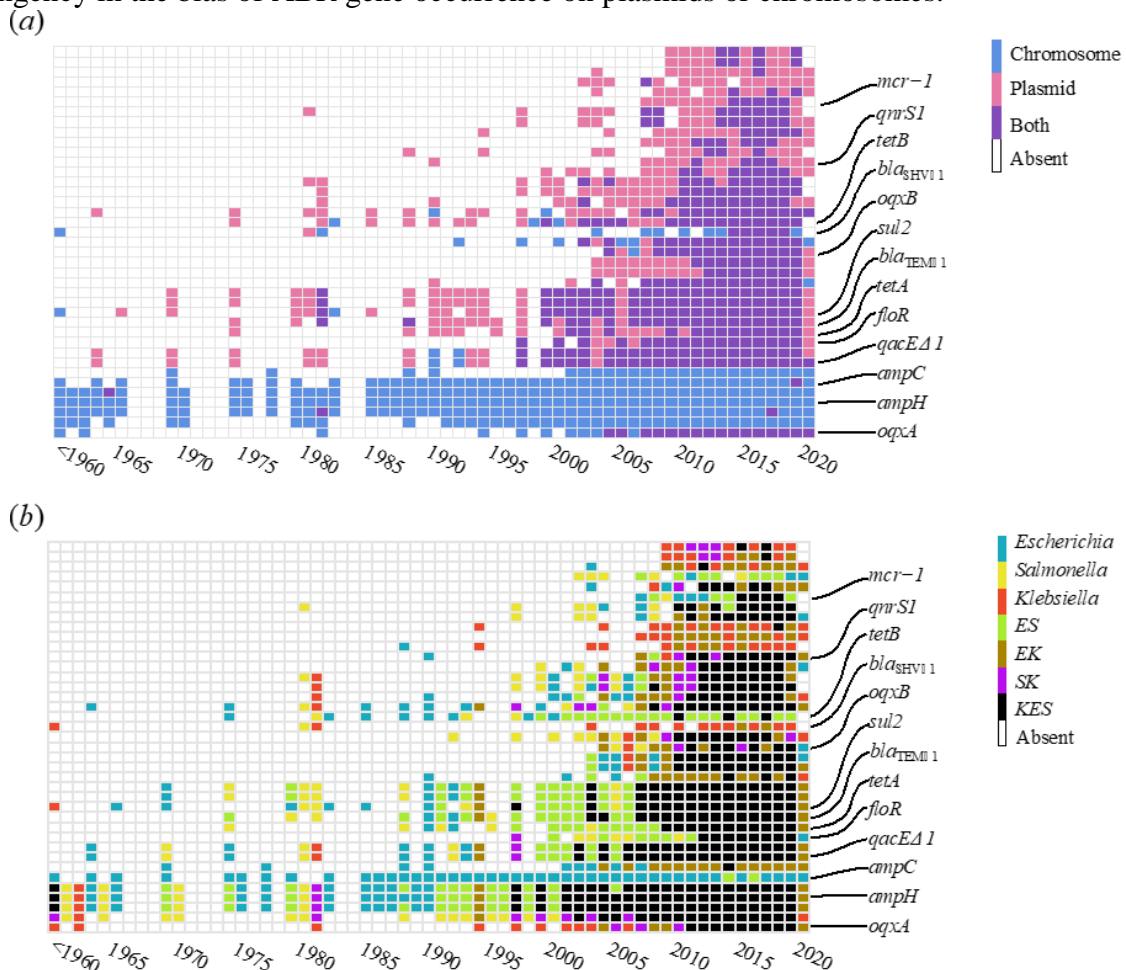
The *mcr-1* gene was the first plasmid-mediated colistin resistance determinant reported, signalling the breach of the last resort polymyxin antibiotics [56]. MCR-1 is a phosphoethanolamine transferase which alters lipid A in the outer membrane of Gram-negative bacteria resulting in reduced affinity to colistin [57]. Homologs of MCR-1 are mainly found on plasmids in all three genera, with several rare homologs on chromosomes in *Escherichia* (1%). Three *Escherichia coli* isolates were identified with *mcr-1* on both replicons. The nearly identical protein sequence of the homologs supports that *mcr-1* is laterally transferred among the genera by plasmids (electronic supplementary material, figure S3e). The gene was first documented on a plasmid in *Escherichia* in 2007 and on chromosome in 2014, in recent years it has been found in all three genera (figure 3).

The gene *sul2* confers resistance against the sulfonamide antibiotics [58,59]. Sulfonamides were among the first synthetic compounds, for which specific antibacterial activities were discovered, and they were also among the first antibiotics to be used for medical treatment. They block the folic acid synthesis in bacteria by competitively inhibiting dihydropteroate synthase (DHPS) [60]. Sul2 is an alternative drug-resistant variant of the DHPS enzyme which replaces the original drug sensitive variant to provide resistance [61]. The gene is mainly found on plasmids and may occur also on chromosomes (6% isolates). A total of 34 isolates encode a *sul2* on both replicons, and the phylogenetic tree of *sul2* indicates multiple transfer events between replicons and across genera (electronic supplementary material, figure S3f). Although typically a plasmid gene, *sul2* was first documented on chromosome in *Klebsiella* in the 1950s and only later in plasmids in *Escherichia* and *Salmonella*; in recent years it has been reported in all three genera (figure 3).

The gene *qnrS1* belongs to the Qnr family of proteins which confer resistance to quinolones and flouroquinolones in Gram-negative bacteria by binding and protecting the target of these antibiotics, type II topoisomerase [62]. The binding of the target seems to destabilize the complex of topoisomerase with the drug resulting in regeneration of the active enzyme [63]. Homologs of *qnrS1* are mainly distributed on plasmids. We identified two isolates that carry this gene on both replicons, including *E. coli* strain LHM10-1 (isolated in 2017 in from China from swine feces) and *K. pneumoniae* strain C2421 (isolated in 2017 in China from human urine sample). The QnrS1 protein sequences are nearly identical in all plasmids and the phylogenetic tree topology indicates lateral gene transfer between replicons and all three genera (electronic supplementary material, figure S3g). It was first documented on plasmids in *Escherichia* and *Klebsiella* in 2007 and in recent years it has been found on both replicons in all three genera (figure 3).

### Temporal pattern of gene presence in replicons supports the phylogenetic inference

To further study the temporal pattern of ABR gene occurrence on specific replicon type – plasmids or chromosomes – we examined the collection date of isolate genomes encoding ABR-genes. The available data reveals two general trends: core ABR genes are typically documented on chromosomes, with only rare findings on plasmids throughout the time range described in the data. Notably, of the 75 core ABR genes that are enriched on chromosomes, 74 (99%) have been documented first on chromosomes, with *oqx<sub>B</sub>* as the sole exception. Additionally, of the 226 genes found to be enriched on plasmids, 208 (92%) were first identified on plasmids. The temporal pattern of plasmid genes shows a trend of early documentation on plasmids and later presence on both replicons, which is in agreement with the phylogenetic trees reconstructed in this study (figure 2). The temporal pattern of ABR gene occurrence as observed in isolate genomes provides further support for the role of historical contingency in the bias of ABR gene occurrence on plasmids or chromosomes.



**Figure 3.** Temporal pattern of ABR gene documentation in replicons and genera. Gene presence according to the isolation date of the strains are summarized in this heatmap. The information of the strains isolated between 1900 and 1960 is condensed in <1960. The figure shows data for 39 genes whose phylogenetic trees were closely examined (ordered as in electronic supplementary material, table S4). The names of 13 genes highlighted in the manuscript are marked. Different colours correspond to gene location. (a) temporal ABR gene pattern according to replicon type. (b) Temporal ABR gene pattern according to genera. ABR gene found in multiple genera are annotated by capitalized abbreviations of the three genera, i.e., E for *Escherichia*, S for *Salmonella* and K for *Klebsiella*.



## Discussion

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In this study, we performed a comprehensive ABR gene analysis across 2,635 complete genome sequences in the related bacterial genera *Escherichia*, *Salmonella* and *Klebsiella* and identified an inverse association between ABR gene prevalence in plasmids and chromosomes. This inverse association strongly suggests that the presence of a homologous gene in the chromosome poses a barrier to plasmid-mediated ABR gene acquisition. Several non-exclusive mechanisms may account for the barrier to lateral gene transfer, as explained in detail below.

The bias of ABR genes towards specific replicon type – chromosomes or plasmids – is clearly associated with the ABR underlying mechanism [7]. ABR genes associated with efflux pumps are primarily encoded on chromosomes, those involved in target alteration can be encoded on both replicon types, and the remaining three mechanisms are almost exclusively encoded on plasmids. ABR genes encoding efflux pumps are frequently part of the core genome (e.g., in the genera we studied here), where they encode for proteins having multiple functions in the cell outside the context of ABR. Examples are export of heavy metals [64], organic pollutants [65], endogenous metabolites [66] and cell signalling molecules [67]. Furthermore, several efflux pumps are important for biofilm formation [68] and bacteria-plant interactions [69]. Core genes are expected to be highly integrated in the cellular transcriptional regulation network and their products are likely well integrated in protein-protein interaction networks. Proteins encoded by the core genome are therefore expected to have a high level of pleiotropic effects on bacterial physiology. Barriers for lateral transfer of core genes are expected to be high, e.g., due to genetic conflicts [7] or the cost of their interference with the well-coordinated cellular processes [23]. The same logic applies to core genes in the other classes of resistance mechanisms, including target alteration and antibiotic inactivation. Core ABR genes that encode for antibiotic target alteration in our dataset include *gyrA*, *parC* and *parE*. These genes may provide resistance to antibiotics that target, e.g., type II topoisomerases, DNA gyrase and topoisomerase IV, which control topological transitions of DNA [70]; all of these are central components in the bacterial information processing mechanisms that are target for drug therapy (e.g., with Fluoroquinolones [4]). The frequency of ABR gene transfer in microbial evolution (i.e., transferability) is therefore expected to depend on the complexity of their function within the cell [18,19] rather than the exact mechanism of resistance, similarly to other genes that do not encode for ABR mechanisms.

So far, we discussed barriers to lateral transfer of antibiotic resistance genes depending on the effect of gene acquisition on the bacterial organism. Notwithstanding, the acquisition of antibiotic resistance gene may have consequences also for the evolution of stably inherited plasmids. Plasmids encoding for antibiotic resistance genes may evolve a stable inheritance in a newly colonized host population also in the absence of selective conditions for antibiotic resistance [71]. In contrast, the presence of selective conditions for antibiotic resistance can maintain in the population plasmids whose inheritance is unstable; such plasmids are at risk of extinction under non-selective conditions [72]. Indeed, plasmids equipped with mobility mechanisms have the capacity to persist via transfer to alternative host populations [73]. Nonetheless, the stability of plasmid vertical inheritance is paramount for plasmid survival and long-term evolution (reviewed in [74]). The potential deleterious effect of ABR gene

acquisition in plasmid genomes to the plasmid fitness [75] may explain the rarity of ABR genes on plasmids (figure 1), specifically in non-mobile plasmids [72].

Plasmid-encoded ABR genes in our dataset are mostly classified as antibiotic target inactivation (figure 1). ABR genes encoding for antibiotic inactivation frequently vary in their relative frequency on plasmids and chromosomes across the three compared genera. Many of those genes correspond to accessory chromosomal genes that are also prevalent on plasmids, indicating that they may be transferred between both replicon types (Table 1*b*). Indeed, accessory genes may prove essential depending on the isolate genetic background and environmental conditions [76], nonetheless, their variable presence in the genus suggests that they are often dispensable. Genes whose evolution is characterized by rapid gain and loss dynamics are expected to encode proteins whose function is weakly dependent on specific genetic backgrounds, and are therefore more likely to evolve by lateral gene transfer [77]. The ABR gene content of plasmids reflects the propensity of specific ABR genes to be successfully transferred among donors and recipients within the plasmid host range.

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**Data accessibility**

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In the supplementary material.

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**Competing interests.**

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The authors declare no competing interests.

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### GENE SHARING AMONG PLASMIDS AND CHROMOSOMES REVEALS BARRIERS FOR ANTIBIOTIC RESISTANCE GENE TRANSFER

#### SUPPLEMENTARY INFORMATION

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## Supplementary Tables & Data

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The following supplementary table and data are available. They can be found online at <https://royalsocietypublishing.org/doi/suppl/10.1098/rstb.2020.0467>

**Supplementary Table S1.** A list of genomes used in this study.

**Supplementary Table S2.** A list of 416 genomes used in the inference of protein families in *Escherichia*.

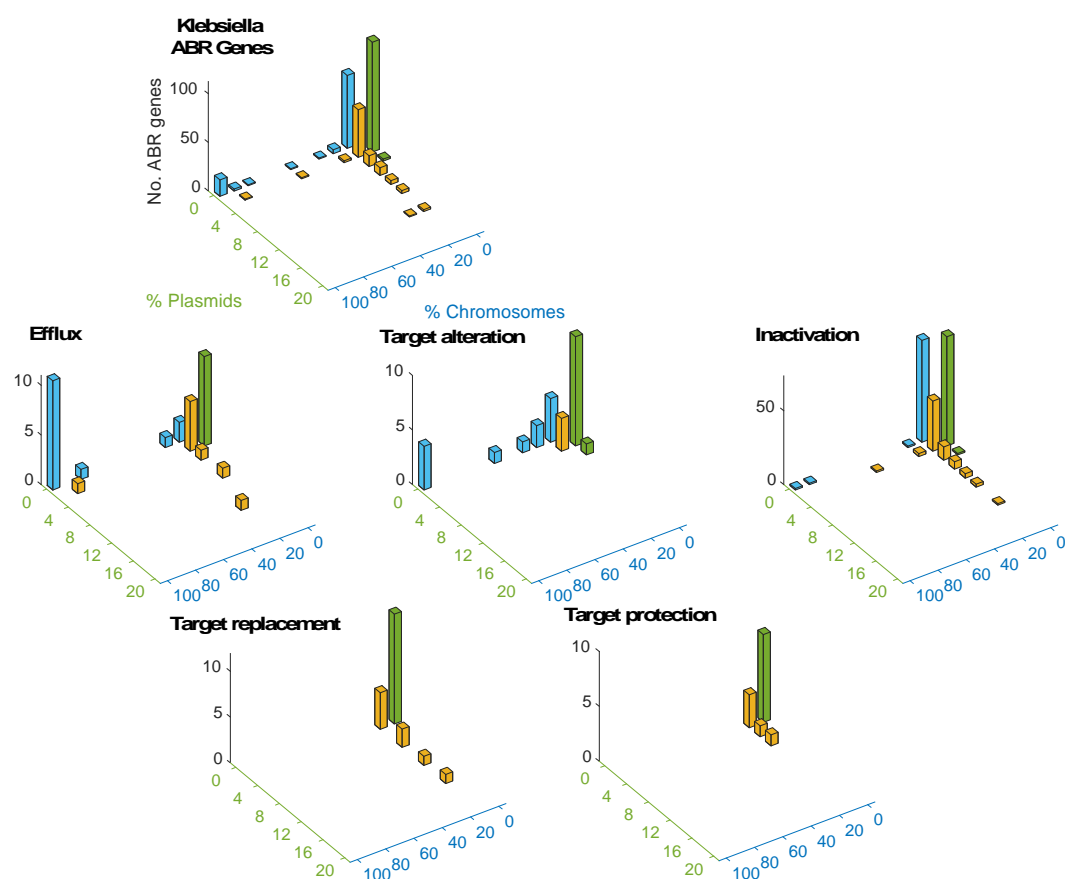
**Supplementary Table S3.** A classification of ABR genes into chromosomal and plasmid genes according to their relative frequency on both replicon types.

**Supplementary Table S4.** A list of ABR genes & isolates where the gene is present on both plasmid and chromosome.

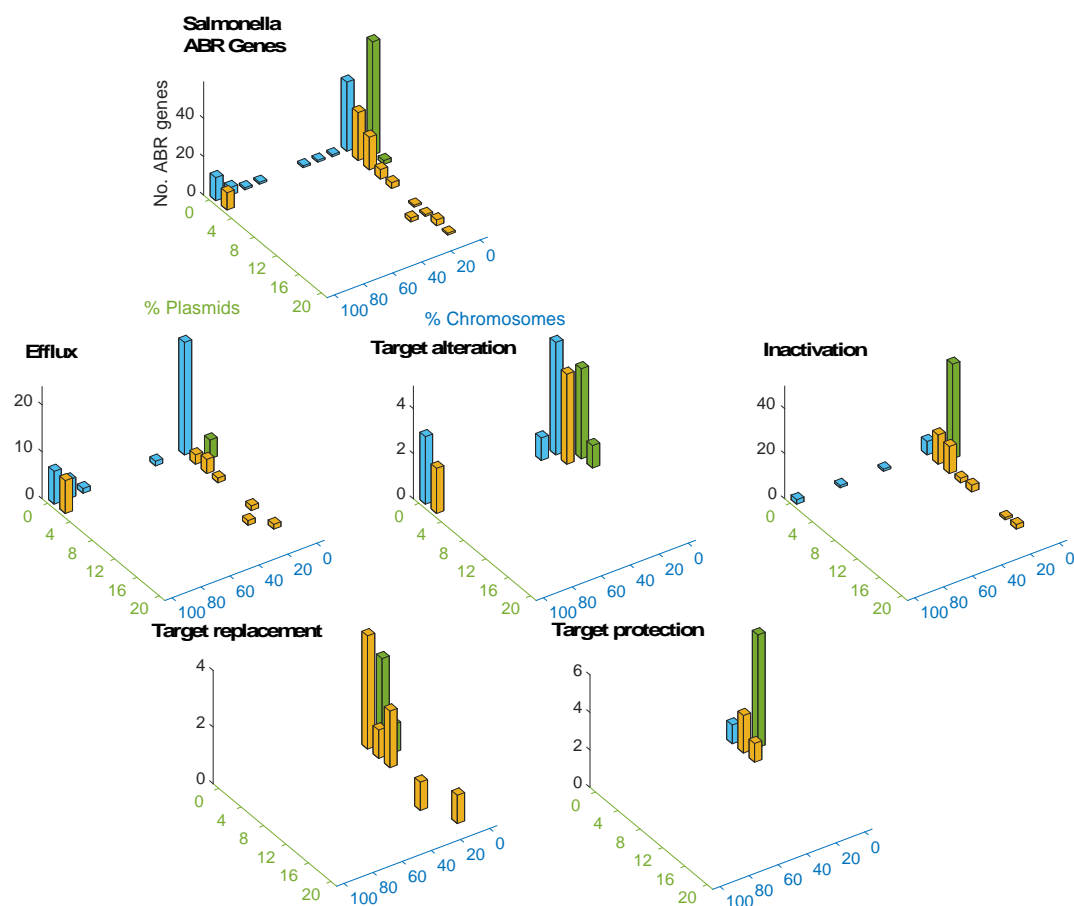
**Supplementary Table S5.** Conclusions of the phylogenetic reconstruction.

**Supporting Data.** Phylogenetic data of 39 ABR gene homologs. For each of the genes, homologs protein sequences, protein information, sequence alignment and phylogenetic tree is supplied.

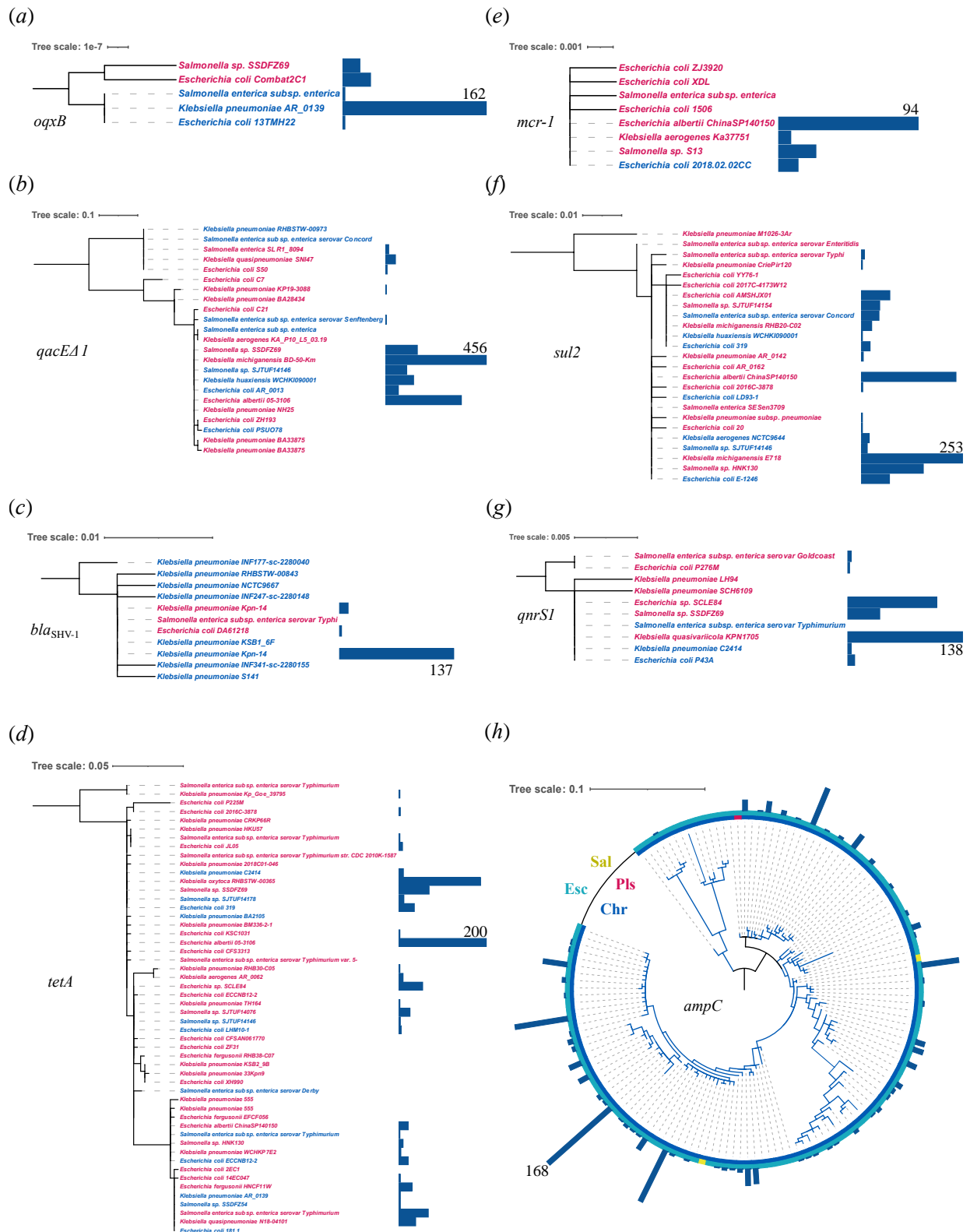
## Supplementary Figures



**Supplementary Figure S1.** A comparison of gene prevalence in plasmids and chromosomes in *Klebsiella* isolates. See extended legend in Fig. 1 (data supplied in Table S2).



**Supplementary Figure S2.** A comparison of gene prevalence in plasmids and chromosomes in *Salmonella* isolates. See extended legend in Fig. 1 (data supplied in Table S2).



**Supplementary Figure S3.** Phylogenetic reconstruction of 8 ABR genes. ABR gene name is written next to the phylogenetic tree. Bars next to the operational taxonomic units show the frequency of redundant amino acid sequences, the highest frequency is noted. *a-g*) Isolate label is colored according to replicon type (plasmid genes in pink and chromosomal genes in blue). *h*) The outer ring shows the genus (*Escherichia* in blue and *Salmonella* in yellow) of ABR gene origin, the inner ring shows whether the gene is encoded on a plasmid or chromosome.

# DISCUSSION

Antibiotic resistance is an evolutionary problem at its core, and a sustainable solution to the problem requires viewing it through an evolutionary lens<sup>1,2</sup>. In this thesis, I have taken such a perspective toward antibiotic treatment and the spread of antibiotic resistance. Using methods in microbiology, experimental evolution, molecular biology, bioinformatics, and advanced statistics I explored several approaches to evolution-informed treatments in the bacterium *Pseudomonas aeruginosa* and studied the drivers of horizontal gene transfer in the Enterobacteriaceae genera *Escherichia*, *Salmonella* and *Klebsiella*. The main findings and insights gained from them are summarized below:

- i. Contrary to general expectations, treatment of *P. aeruginosa* strain PA14 with multiple  $\beta$ -lactam antibiotics did not result in treatment failure. Instead, the  $\beta$ -lactam treatments were successful in eradicating a significant proportion of bacteria. The  $\beta$ -lactam only sequence containing carbenicillin, doripenem, and cefsulodin even outperformed the heterogeneous sequences. This goes to show that the available antibiotics offer unexplored, highly potent treatment options that can be harnessed to counter the spread of drug resistance (**Chapter 2**).
- ii. The success of the carbenicillin, doripenem, and cefsulodin sequence was attributable to the low rate of resistance acquisition to doripenem and the low frequency of cross-resistance to it. These results further highlight the importance of evolutionary trade-offs such as reduced cross-resistance in treatment design. They also introduce spontaneous resistance rates of component antibiotics as an adaptive constraint enhancing the efficacy of sequential treatments (**Chapter 2**).
- iii. The collateral responses of the spontaneous mutants derived from the fluctuation assay were different than that of the long-term evolved populations; this was the case for both, bacteria resistant to the  $\beta$ -lactam antibiotics and to ciprofloxacin. Further, in the case of the  $\beta$ -lactam antibiotics, the collateral effects of the mutants were important predictors of the efficacy of fast sequential  $\beta$ -lactam therapy and not those of the long-term evolved populations. This raises the question - do evolutionary trajectory-dependent collateral profiles dictate the choice of sequential treatment? (**Chapters 2 and 3**).
- iv. Resistance to ciprofloxacin provided consistent, conserved collateral sensitivity towards the aminoglycoside antibiotics gentamicin and tobramycin across genomic diversity in *P. aeruginosa*. This makes the trade-off a good candidate for an adaptive constraint in sequential and combination treatments of *P. aeruginosa* infections (**Chapter 3**).
- v. Sequential and combination treatments with piperacillin/tazobactam and gentamicin were able to constrain adaptation in a mixed population of *P. aeruginosa* displaying predominantly negative social interactions. The efficacy of these evolution-informed treatments was potentiated by the antibiotic piperacillin/tazobactam. Further, the speed of adaptation differed between the

mixing and no-mixing meta-treatments. Evolution-informed treatments can thus be considered on a mixed population of *P. aeruginosa* potentially contingent on the nature of interactions in the population (**Chapter 4**).

- vi. The distribution of antibiotic resistance (ABR) genes differed between plasmids and chromosomes in the genera *Escherichia*, *Salmonella*, and *Klebsiella*. The distribution further varied with the antibiotic resistance mechanism. The degree of integration into the cellular network appeared to be the determining factor behind the distribution (**Chapter 5**).

In the following sections, I will frame these insights considering the current literature and outline emerging research directions. I will then give a final perspective on how to translate these insights into the clinic and their contribution to basic research. Finally, I then end the discussion with some concluding remarks on future research foci on evolution-informed treatments.

### **Treatment with $\beta$ -lactam-only sequences**

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We found that  $\beta$ -lactam sequences are at least as potent at eradicating bacteria as sequences of antibiotics that target distinct cellular functions. Although this result is counterintuitive, it is not completely unexpected. Combination treatments with  $\beta$ -lactam antibiotics only have shown some success in the past<sup>3</sup>. Simultaneous treatment with ticarcillin and ceftazidime was demonstrated to be highly efficacious in a rat peritonitis model<sup>4</sup>. In the treatment of bacterial soft tissue infections, the combination of cefotaxime and mecillinam did better than the tested monotherapy<sup>5</sup>. More recent, evolution-oriented studies found that a triple combination of meropenem, piperacillin, and tazobactam was able to constrain resistance evolution in methicillin-resistant *Staphylococcus aureus* (MRSA)<sup>6</sup> and that the combination of cefotaxime and mecillinam was effective against the CTX-M-15  $\beta$ -lactamase of *Salmonella enterica*<sup>7</sup>. The triple combination was shown to be synergistic while collateral sensitivity was described between the antibiotics in both studies. Our results, along with the studies mentioned above support the notion that treatment with multiple  $\beta$ -lactam antibiotics can be effective and show that there is more in our treatment armamentarium than previously considered.

The spontaneous rate of resistance, frequency of cross-resistance, and rate of antibiotic switching were determined to be strong predictors of the success of triple  $\beta$ -lactam sequential therapy. The rate of switching has been implicated in sequential therapy success in the lab previously; fast-switching treatments with antibiotics having distinct modes of action were shown to outperform the monotherapy and even the slower switching treatments<sup>8,9</sup>. Fast-switching between environments limits the time the population spends in any environment thereby potentially limiting the selection and spread of resistant mutants. The low frequency of cross-resistance between the sequentially alternating antibiotics increased bacterial extinction. A lack of cross-resistance is related to collateral sensitivity as mutant bacteria resistant to an antibiotic maintain ancestral levels of sensitivity to another. Therefore, our results are consistent with studies that demonstrate the importance of collateral sensitivity for the success of sequential therapy<sup>10–19</sup>. It is important to note that collateral sensitivity may still play a role in our setup, only that we didn't directly test for it. Further, our results come with a sense of



irony, as a lack of cross-resistance was observed to constrain adaptation in a triple  $\beta$ -lactam sequence which is generally avoided due to the fear of cross-resistance.

The inclusion of antibiotics with a low rate of spontaneous resistance was significantly associated with treatment success. The spontaneous resistance rate is the intrinsic rate of resistance on an antibiotic for a bacterium. It can vary with the tested antibiotic<sup>20,21</sup> contingent on factors such as available mutational space, induction of the bacterial SOS response, DNA content, etc<sup>22</sup>. To date, low spontaneous resistance rates have only been associated with resistance emergence in monotherapies. As a consequence, research in spontaneous resistance rates has focused on trying to reduce the rate, either by identifying antibiotics targeting multiple pathways<sup>23,24</sup> or by using adjuvants that target the SOS response<sup>25,26</sup> to reduce evolved resistance. Our study extends the role of the resistance rate of antibiotics by identifying it as an adaptive constraint that can aid the potency of sequential treatments.

The three predictors mentioned above can theoretically interact. For example, if these predictors were to be combined, then fast antibiotic switching should further limit the likelihood of resistance acquisition on doripenem. When the antibiotic is switched, resistance to it may evolve but it is unlikely that there is cross-resistance to doripenem. As a consequence, when the treatment eventually switches back to doripenem, the bacteria would still be sensitive to it and have a higher probability of extinction. We could not find an association between these three predictors with our GLM. Further evolution experiments with more replication might be necessary to disentangle the main and interactive effects of these predictors. Many additional open questions remain which could be answered with these experiments – Is the association between these predictors and treatment success causative? As of now we only have correlational data between the predictors and treatment success, it is necessary to show that directed variation in resistance rates can result in treatment success for causation. These experiments should also be conducted with other antibiotics which can target the same or distinct pathways. This would give us a broader perspective on the variation in resistance rates and how they affect treatment outcomes. Moreover, we have only tested this treatment strategy in the *P. aeruginosa* type strain PA14. In an infection, however, many clone types can be present and the resistance rate is likely influenced by the genomic background<sup>27–30</sup>. To determine resistance rates that can constrain adaptation in a conserved manner, the above experiments could be done with the major *Pseudomonas aeruginosa* clone type (mPact) panel<sup>31</sup>. Such a stratified study would also answer the question of whether there is a threshold for resistance rate above which it can no longer constrain adaptation. We could then further integrate the data for these predictors for the mPact panel with data on negative hysteresis for the panel (unpublished data) to assess the relative quantitative contribution of these predictors to sequential therapy success. Finally, it will also be worthwhile to test if spontaneous resistance rates also act as an adaptive constraint in combination treatments.

### **Matching collateral profiles and intended treatment**

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We found that the collateral responses differed between mutants derived from the fluctuation assay and long-term evolved populations. This was the case on doripenem and piperacillin/tazobactam. While long-term evolved populations on carbenicillin and cefsulodin

were all resistant to doripenem<sup>32</sup>, the corresponding mutants had a low frequency of cross-resistance to it. Similarly, ciprofloxacin resistance in long-term evolved populations gave collateral sensitivity to piperacillin/tazobactam but most of the ciprofloxacin resistant mutants were cross-resistant to the antibiotic. The mutants and the long-term populations varied in several ways that could affect the evolved collateral response. The mutants on both antibiotics were grown in minimal medium without any antibiotic and then later selected on agar with a low concentration of the antibiotic. Any mutation not incurring a high fitness cost could be selected in this scenario. The long-term populations were grown over a longer time in increasing concentration of the antibiotic. The bacteria, to survive either required a single large effect mutation or multiple small effect mutations. Further, the long-term ciprofloxacin resistant populations were propagated on agar and not liquid medium, which added structure to the population and likely reduced access to the antibiotic, in turn affecting evolved mutations. Difference in evolved mutations could result in different collateral effects. Indeed, collateral responses have been shown to change with time<sup>33</sup>, with the concentration of the selecting antibiotic<sup>34</sup>, and with the mode of growth<sup>35</sup>. More importantly, there were consequences to differing collateral responses. The success of the  $\beta$ -lactam sequential therapy was predicted by the collateral responses of the mutants but not the long-term populations. In hindsight, this makes sense. The length of the growth season and the concentration of antibiotic in fast sequential therapy is similar to that in the fluctuation assay. Therefore, the expected mutations in the growth season should be similar to those from the fluctuation assay. As a consequence, the collateral responses of these mutations should govern the success of fast sequential therapy. This raises the question - should evolutionary trajectory-dependent collateral profiles be matched to the choice of sequential treatment? Experiments are currently underway to test this hypothesis. If true, this would add another layer of complexity to the clinical translation of collateral sensitivity.

### **Robust collateral sensitivity due to ciprofloxacin resistance**

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Using the mPact panel<sup>31</sup>, we demonstrated that resistance to ciprofloxacin resulted in collateral sensitivity to the aminoglycosides gentamicin and tobramycin, across genomic diversity and evolutionary trajectory in *P. aeruginosa*. We chose to test this particular trade-off because previous work in the lab showed that it was robust across replicates of the strain PA14<sup>32</sup>. There are other studies that have hinted at its potential robustness. Resistance to ciprofloxacin was observed to result in robust hypersensitivity to one or more aminoglycosides in clinical isolates of *E. coli*<sup>36</sup> and *P. aeruginosa*<sup>37–39</sup>. While these studies covered some sequence types, it is unclear how representative they are of the species-wide diversity. Two more studies characterized collateral responses to ciprofloxacin resistance. The first looked at collateral responses of specific mutations in the *gyrA* and *parC* alleles in *Streptococcus pneumoniae* that conferred resistance<sup>40</sup>. They found consistent sensitivity to gentamicin irrespective of the tested mutant. The second study tested if different clinical mutations reconstructed in strain PA14 of *P. aeruginosa* could interfere with the evolution of collateral sensitivity to tobramycin and aztreonam on ciprofloxacin resistance<sup>17</sup>. They found that most genotypes evolved collateral sensitivity to these antibiotics. Taken together, all these studies show the robustness of this trade-off against the tested clinical variation which covers some genomic variation in the

species. Genomic variation can have massive consequences on the robustness of collateral sensitivity. A study attempting to delineate collateral responses across the ESKAPE pathogens could not find any conserved trade-offs across the 6 tested species. At best, only 3 of the 6 species showed the same trade-off<sup>41</sup>. Our study tests the robustness of collateral sensitivity due to ciprofloxacin resistance across genomic variation covering the entire species. This is important because infecting bacteria could come from a wide range of backgrounds<sup>27–29</sup>, and therefore a wide variation in genomic backgrounds must be tested for robustness. Our results, along with those mentioned above support the consideration of this trade-off for further clinical translation.

Why is collateral sensitivity to the aminoglycosides observed so consistently upon ciprofloxacin resistance? Resistance to ciprofloxacin can evolve through several routes like changes in the DNA Gyrase, Topoisomerase IV, the efflux machinery, outer membrane diffusion channels, in genes such as *mutS*, *mutL*, and *nuoD*, and plasmid-based inactivating enzymes<sup>42–44</sup>. Some of these routes can also confer resistance to the aminoglycosides such as upregulation of the MexXY-OprM efflux pump and the inactivating enzyme *aac(6')-Ib-cr*<sup>45,46</sup>. Yet, these do not appear to be selected frequently. Instead, commonly selected mutations include those in DNA Gyrase, Topoisomerase IV, and specific efflux pumps/their regulators<sup>17,36–38,41</sup>. What could be causing this bias in selection targets? The mutability of genes can differ and is contingent on many factors such as copy number, length of gene, sequences surrounding relevant bases for the resistance phenotype, etc<sup>22</sup>. This could be the reason for a specific set of genes being selected frequently. Although less common, other mutations are selected which also confer collateral sensitivity to the aminoglycosides<sup>13,32,47</sup>. Genomics of the large set of resistant mutants and populations generated in this study could help identify more accurately conserved mutations conferring sensitivity.

Even though we identified conserved collateral sensitivity, it is still not known if it would successfully constrain resistance evolution. Followup evolution experiments directly manipulating the treatment sequence to maximize collateral sensitivity would be needed to address that. These are currently underway. Once conserved mutations conferring collateral sensitivity have been identified, one could ask the question of what factors might predispose these genes to mutation. This would not only add to basic knowledge of mutational bias but could also be used to better predict the expected mutations on antibiotic treatment and the associated chance of sensitivity. Conserved mutations could also serve as markers for treatment using collateral sensitivity irrespective of ciprofloxacin treatment. Further, it would also be interesting to know why such different mutations all give conserved collateral sensitivity to the aminoglycosides.

### **Evolution-informed treatment with a mixed population of a single species**

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Sequential and combination treatments using piperacillin/tazobactam and gentamicin outperformed both monotherapies in the mixing meta-treatment and the gentamicin monotherapy in the no-mixing meta-treatment. The effect of social interactions on resistance evolution has been assessed before, however, it has focused on interspecies interactions under monotherapy. The effect of the interaction usually depends on the exact interaction under study.

Negative interactions, which reduce the survival of one or both interacting partners, usually counter the *de novo* evolution and spread of resistance mutations<sup>48–51</sup>. When these interactions are eliminated, for example, due to the elimination of the competitors due to higher antibiotic concentration, the resistant variant spreads<sup>48</sup>. Positive interactions, which increase the survival of one or both interaction partners, can constrain or speed up resistance evolution. Cross-feeding interactions reduce the resistance of the network to that of the weakest link<sup>52</sup>. As a consequence, under antibiotic treatment, the constituent members always ‘wait’ for each other to evolve. This slows down resistance evolution compared to a monoculture<sup>53</sup>. Antibiotic-inactivating enzymes speed up resistance for both, its producers and surrounding non-producers who benefit from it. Exposure to ampicillin increased the frequency of  $\beta$ -lactamase producing *E. coli*, thereby ensuring the survival of the population<sup>54</sup>. In another study, co-culture of *P. aeruginosa* with carbapenemase-producing *Stenotrophomonas maltophilia* accelerated the *de novo* evolution of imipenem resistance in *P. aeruginosa*<sup>55</sup>. This was likely due to an increased mutational supply because of the protection provided by the carbapenemase. A very recent study from the same lab tested the response of co-culture of *S. maltophilia* and *P. aeruginosa* to combination treatments with ciprofloxacin, tobramycin, and colistin. They found that antibiotic resistance evolved in all treatments but the exact composition of the community varied with the treatment. Usually, the more resistant of the species to the antibiotic in question dominated<sup>56</sup>. Our study demonstrates that interactions in a mixed population of a single species can also affect resistance evolution. It further confirms the efficacy of evolution-informed treatments in a mixed population dominated by negative interactions. Finally, it highlights factors that can affect efficacy such as the use of piperacillin/tazobactam and the choice of meta-treatment.

Why do piperacillin/tazobactam and the choice of meta-treatment affect treatment efficacy? In earlier studies from the lab, strain PA14 was able to adapt to piperacillin/tazobactam monotherapy<sup>32,57</sup> although the magnitude of evolved resistance was on the lower side compared to other antibiotics<sup>32</sup>. Further, recent unpublished data in the lab suggests that the resistance rate on piperacillin/tazobactam is relatively lower than other antibiotics (See also Barbosa et al. 2018 PLoS Biol, suppl fig. 10) across members of the mPact panel (unpublished data). Thus, it could be that like doripenem, adapting to piperacillin/tazobactam is hard due to a dearth of evolutionary solutions on the antibiotics. Still, the striking lack of growth in the no-mixing treatment begs additional factors which explain its efficacy. Community context has been shown to constrain adaptation in various environments, including antibiotics<sup>49,58,59</sup>. Further, the presence of a community has been observed to increase the cost associated with a resistance gene<sup>48</sup>. This effect of community was mediated by competitive interactions as they increase the cost of adaptation. Thus, it is plausible that the negative interactions observed in our mixed population made adaptation to piperacillin/tazobactam difficult. But why was adaptation constrained on piperacillin/tazobactam alone? The cost of resistance genes can vary between antibiotics<sup>60</sup>. Perhaps the cost of resistance on piperacillin/tazobactam is higher than on gentamicin. At least in strain PA14, the maximum yield of piperacillin/tazobactam resistant populations was lower than that of gentamicin resistant populations<sup>32</sup>. Competition could then exacerbate this cost which results in resistance being constrained and instead an alternative strategy of survival –

tolerance being chosen. The mixing meta-treatment allows for a higher mutational supply and as a consequence low-cost mutations can be selected allowing for resistance. Alternatively, this treatment could have encouraged horizontal gene transfer by promoting strain retainment. The horizontal transfer of genes then further could have led to the spread of a low-cost resistance gene which was lost in the no-mixing treatment. Genomics of the adapted populations would help shed light on these hypotheses.

While our study shows that evolution-informed treatments with tested antibiotics can work, the exact factors contributing to their success must be delineated. Major open questions remain such as, what causes the particular efficacy of piperacillin/tazobactam? Do negative hysteresis, collateral sensitivity, and spontaneous resistance play a role? What feature of the mixing treatment contributes to faster adaptation? Is the outcome consistent no matter what the interactions between the members of the population are? What are the targets of selection? Is there horizontal transfer of genetic material between the different strains? Does it contribute to adaptation? Understanding the reason for the success of these treatments would not only help us tailor the evolution-informed treatments to expected infection conditions but might also help identify more adaptive constraints that aid the potency of such treatments.

### **Drivers of horizontal transfer of antibiotic resistance genes**

We found that the distribution of ABR genes between the plasmid and chromosome varied contingent on the resistance mechanism class to which they belonged, in the genera *Escherichia*, *Salmonella*, and *Klebsiella*. Genes belonging to antibiotic efflux were enriched on chromosomes while those belonging to target inactivation, replacement, and protection were enriched on the plasmid. Genes encoding altered target proteins were distributed on both. ABR genes such as efflux pumps form a part of the core genome of the species studied here. This is because they tend to play roles essential for the core physiology of the cell such as the export of heavy metals<sup>61</sup>, organic pollutants<sup>62</sup> and endogenous metabolites<sup>63</sup>, and cell signaling molecules<sup>64</sup>. Furthermore, several efflux pumps are known to be important for biofilm formation and bacteria–plant interactions<sup>65</sup>. Core genes and their products are expected to be highly integrated into cellular networks and have many pleiotropic effects. Their loss could interfere with well-coordinated cellular processes<sup>66</sup>. Therefore, they are enriched on the more stable replicon, the chromosome. Other genes restricted to chromosomes in our dataset such as *gyrA*, *parC*, and *parE* are also core chromosomal genes with roles in maintaining DNA topology. We conclude that the degree of connectivity of ABR genes impacts their localization and therefore their transferability. Our results are in line with the previously proposed complexity hypothesis which identifies the degree of connectivity determining the transfer of genes overall<sup>67</sup>

### **Final perspectives**

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#### **I. Clinical translation of insights gained from the thesis**

The insights from this thesis cover different aspects of evolution-informed treatments. Some of the insights focus on adaptive constraints with the aim of improving evolution-informed treatments. Other insights highlight the considerations for the application of these treatments to infections caused by a mixed population/community of bacteria. In this section, I discuss

what more could be done to bridge the gap between these experimental insights and their clinical use.

Spontaneous resistance rate as an adaptive constraint is a promising candidate for clinical translation given the underlying molecular mechanisms need not be known to apply it in therapy. Clinical translation of this adaptive constraint requires knowledge of the resistance rate of a given organism-antibiotic combination. While generating such information in the lab for distinct lab strains and clinical isolates<sup>68,69</sup> provides baseline data to test hypotheses on the spontaneous resistance rate; the determined rates might not be the same in a patient due to differences in cost of the mutations, population size, rate and pattern of antibiotic usage, etc<sup>70</sup>. Information on meaningful rates in patients instead could be derived from clinical trials of the antibiotics where resistance emergence in patients is noted<sup>71</sup> and a meta-analysis conducted to identify antibiotics with low rates of resistance<sup>72</sup>. Information on the spontaneous rates of resistance could then be combined with information on collateral sensitivity between antibiotic pairs determined with clinical isolates and fast sequential switching to design sustainable antibiotic treatments which can be tested in the clinic.

The clinical utility of collateral sensitivity has been questioned a lot, in part because of its strong dependence on the exact mutation conferring resistance and the subsequent heterogeneity arising in replicate populations. Resistance to ciprofloxacin results in conserved collateral sensitivity to the aminoglycosides gentamicin and tobramycin across genetic diversity in *P. aeruginosa* our dataset. Our results are in line with other studies in *P. aeruginosa* that show that ciprofloxacin resistance confers consistent collateral sensitivity to the aminoglycosides amikacin<sup>38</sup>, tobramycin, and the monobactam aztreonam<sup>39</sup> across different clone types. These studies also show that the conserved phenotype arises from different mutations in a small number of genes. Although this trade-off is not consistent across different species<sup>41</sup>, it is still valuable as a candidate for clinical translation in the treatment of infections dominated by *P. aeruginosa* such as adult cystic fibrosis<sup>73</sup>. For its clinical translation, the expression and stability of this trade-off would need to be demonstrated in a large number of clinical isolates from patients having undergone ciprofloxacin treatment. A switch to one of the collaterally sensitive antibiotics could then be tried to see if resistance evolution is constrained. It is important to keep in mind that a single switch would need to be tried as information on the trade-off is only available in one direction. Collateral response to ciprofloxacin due to resistance on gentamicin/tobramycin/amikacin/aztreonam would need to be characterized before attempting treatment with multiple switches. Further, one could sequence the clinical isolates resistant to ciprofloxacin displaying collateral sensitivity; this would help identify the targets of selection and therefore help to confirm molecular markers of the phenotype. This in turn would facilitate identifying the presence of the trade-off in infections from sequence data alone. It is worth noting that ciprofloxacin is not a preferred drug for treatment because of its severe side effects. It may still be valuable when it is inhaled, as this may reduce side effects and concentrate antibiotic in the tissue of interest, especially in comparison to IV treatment. While inhalable ciprofloxacin is not yet available commercially, recent clinical trials demonstrate a trend in favour of its efficacy<sup>74</sup>

Sequential and combination treatments with piperacillin/tazobactam and gentamicin were able to constrain adaptation in a mixed population of *P. aeruginosa* dominated by negative interactions. This is an important first step in the direction of clinical testing of these treatments for infections with mixed populations of *P. aeruginosa*. It further highlights some considerations for the treatment of multi-strain/species infections. The potency of the evolution-informed treatments was increased by the antibiotic piperacillin/tazobactam. This effect of the antibiotic could be independent of the social interactions present in the population or it could be an interaction between the antibiotic and the social interactions present (negative interactions could make it harder to adapt to it, please see above). If it is independent of the interactions present, then the level of treatment personalization needed would be minimal. If it is dependent on social interactions between bacteria, however, then how different types of interactions at the population level affect the treatment would need to be determined. This would allow us to establish in which cases the treatment strategy would work. For treatment purposes, we would still need to be able to assess interactions in a patient sample fast enough for treatment. This could be achieved through meta-genomic approaches assessing for traits involved in interactions such as bacteriocins, type 6 secretion systems, siderophores, etc<sup>75,76</sup>.

## **II. Contribution of results to basic research**

The approach to most of the different studies included in this thesis was from a treatment standpoint, however, the insights gained from the results further knowledge also from the perspective of basic research.

We found that a low spontaneous rate of resistance of the constituent antibiotics and a low frequency of cross-resistance between them could constrain adaptation when switching between them. The spontaneous rate of resistance to an antibiotic is different from the mutation rate of the cell. The mutation rate is the rate of mutation per nucleotide, per locus, or, eventually, for the whole genome that occurs per cell division<sup>77</sup>. The spontaneous rate of resistance, as determined in a fluctuation assay, is the rate of emergence of phenotypic resistance. It is a measure of all the mutants in a population irrespective of whether they arose by independent mutation or are derived from each other<sup>22</sup>. It is linked to the mutation rate, as the mutants arising are still due to the basal error rate of the DNA polymerase. However, the mutations will only be selected, and therefore observed, if they occur in resistance genes. Therefore, the spontaneous rate of resistance also depends on the number of likely evolutionary targets for resistance to the antibiotic in question. Given that the  $\beta$ -lactam antibiotics used in the experiment are not known to modulate the mutation rate we can assume that it did not differ between the three antibiotics in the fluctuation assay. Then what must differ is the number of likely evolutionary targets for resistance. The low spontaneous rate of resistance on doripenem implies that there are not many likely mutational solutions on this antibiotic. Further, the lack of cross-resistance to it indicates that mutations in response to other antibiotics did not confer fitness on doripenem. What does this mean for fluctuating selection in general? Fluctuating selection complicates adaptation by changing the direction of selection on a phenotype in short intervals of time<sup>78</sup>. Adaptation can further be retarded in fluctuating conditions if the switching environments are related due to evolutionary trade-offs, negative hysteresis, and/or if the rate of fluctuation is fast, or irregular<sup>79</sup>. Our results show that adaptation can further be constrained if the number of evolutionary solutions on one or more of the fluctuating environments is

limited. Moreover, they emphasize that the evolutionary success of the mutation does not only depend on its effect on the currently selected trait but also on trade-offs that determine fitness in future selective environments, something that is usually not taken into consideration when assessing mutation rates/spontaneous mutants. The insights from the study, therefore, add to the field of fluctuating selection.

Resistance to ciprofloxacin gave conserved collateral sensitivity to the aminoglycosides gentamicin and tobramycin across genomic diversity in *P. aeruginosa*. This trade-off appears to extend to all clinically relevant aminoglycosides for this species as recent studies showed collateral sensitivity to amikacin and tobramycin upon ciprofloxacin resistance<sup>38,39</sup>. These studies also showed that collateral sensitivity always arose due to mutations in a limited number of genes, in specific, those coding for gyrase, topoisomerase, and efflux. Bias towards these genes could arise from several reasons<sup>22</sup>. Elucidating the cause behind it would add to basic knowledge on mutational bias. Further, all these mutations result in the phenotype of collateral sensitivity. Understanding how different mutations all result in the same phenotype would increase our understanding of the mechanistic basis of collateral sensitivity.

Evolution-informed treatments were able to constrain adaptation in a mixed population of *P. aeruginosa*. Therefore, fluctuating selection and simultaneous application of selective pressure can constrain the adaptation of a mixed population of bacteria and potentially even a community. Since we do not yet know what factors can explain this efficacy, the contribution of these results to basic research outside of the above remains speculative. If the efficacy of piperacillin/tazobactam was due to the antibiotic only, for example, due to low spontaneous resistance, directional negative hysteresis, collateral sensitivity, or synergy, then it would mean that these adaptive constraints can also constrain adaptation in a mixed population both in antibiotic environments but also likely in others. However, the efficacy of piperacillin/tazobactam could also be due to the interactive effect of the antibiotic and the negative social interactions present in the bacterial population. This would indicate that in addition to constraining adaptation in constant environments, negative interactions amongst bacteria can increase the selective effect of fluctuating and simultaneous selective environments to constrain adaptation even further under them. It could also be that negative interactions heighten the selective pressure due to one or more of the selective constraints. Further experiments to tease apart the effects of the constraints and the interactions will be necessary to resolve this.

### **Concluding remarks**

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In this thesis, I looked at antibiotic treatment and spread from an evolutionary perspective. In doing so, I, along with the help of others was able to identify a low spontaneous rate of resistance to an antibiotic as a new adaptive constraint aiding evolution-informed treatment, demonstrate the robustness of an existing collateral sensitivity trade-off across genomic diversity and evolutionary trajectory in *P. aeruginosa*, show that evolution-informed treatments can constrain adaptation in a mixed population, and finally, implicate the degree of connectivity of an antibiotic resistance gene in cellular networks as a factor affecting its lateral transfer. I then further outlined the research directions emerging from the results presented in



this thesis and gave a final perspective on how the insights from the thesis could be translated into the clinic and their contribution to basic research.

Subsequent research into evolution-informed treatments should focus on at least three broad foci. Firstly, potential candidates from existing adaptive constraints must be identified which display a tendency for a robust phenotype in clinical settings. Their robustness must then be demonstrated so that they can be considered for clinical use. Even if complete robustness is not observed, constraints displaying a high probability for robustness should be considered. Second, research should be directed towards evaluating evolution-informed treatments with clinically relevant factors such as the presence of an infecting community, host microbiome, pharmacokinetic changes in drug concentration, etc. This would help identify variables that can affect treatment success and potentially more adaptive constraints. Third, efforts must be made to delineate where personalization of therapy (precision medicine) is necessary. Once this has been done, research can be directed towards making these personalized diagnostics achievable within the treatment span and also affordable for the patient. Research on the above-mentioned foci should further combine methods from many disciplines; integrating evolution experiments, bioinformatics, machine learning, mathematical modeling, and molecular biology will allow for insights not possible from any discipline alone<sup>80,81</sup>. Concomitant with research into evolution-informed treatments, concerted efforts to introduce them into daily clinical practice must be made. While combination treatments are utilized in the clinic<sup>3,82</sup>, not many other evolution-informed treatments have made it to clinical practice. Resistance evolution to antibiotic treatments is not only predicted by evolutionary theory but also documented in the clinic<sup>71,83,84</sup>. Evolution-informed treatments must be tested alongside the current standard of care and if found to be non-inferior, be used in clinical practice. Only if we target antibiotic resistance at its evolutionary core can we find a sustainable way out of the problem.

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