Toward a quantitative understanding of antibiotic resistance evolution

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The rising prevalence of antibiotic resistant bacteria is an increasingly serious public health challenge. To address this problem, recent work ranging from clinical studies to theoretical modeling has provided valuable insights into the mechanisms of resistance, its emergence and spread, and ways to counteract it. A deeper understanding of the underlying dynamics of resistance evolution will require a combination of experimental and theoretical expertise from different disciplines and new technology for studying evolution in the laboratory. Here, we review recent advances in the quantitative understanding of the mechanisms and evolution of antibiotic resistance. We focus on key theoretical concepts and new technology that enables well-controlled experiments. We further highlight key challenges that can be met in the near future to ultimately develop effective strategies for combating resistance.

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Introduction

Progress in our quantitative understanding of the evolutionary dynamics leading to antibiotic resistance holds promise to help avert the looming resistance crisis [1]. While changes in antibiotic prescription strategies can contribute to countering resistance [2], optimized treatment schemes that take into account the dynamics of resistance evolution are urgently needed. The best-known mechanisms of antibiotic resistance commonly found in the clinic or laboratory include antibiotic degrading enzymes, drug target modification, efflux, and the prevention of drug uptake [3–6]. These mechanisms have been characterized in great detail in decades of fruitful work, culminating in databases of the 'resistome'—the collection of all known genes conferring resistance [7–9].

More recently, transcriptomic studies have provided a useful intermediate phenotypic description of resistance, showing that information on global gene expression can improve predictions of the resistance phenotype compared to genotypic data alone [10,11].

The resistance of a bacterium to a drug is determined by measuring the minimal inhibitory concentration (MIC), that is, the lowest concentration that completely inhibits growth of a clonal culture [12]. An increase in resistance occurs when the population can grow in higher concentrations of antibiotic. Resistance is a genetically inherited trait, acquired by bacteria through one of two main processes: spontaneous *de novo* mutations and horizontal gene transfer [13,14]. Still, the level of resistance is often not entirely determined genetically, but can be heterogeneous within a population, depend on the environment, on the population structure, or on the physiological state of the cell [15–18]. In this review, we focus on specific examples where population dynamics and cell physiology affect drug sensitivity, and on quantitative aspects that determine the emergence and spread of *de novo* resistance mutations. We particularly emphasize recent studies that combined experiments and theoretical modeling. Related influential studies on collective resistance and on the effect of drug combinations on resistance evolution have been reviewed elsewhere [19–23].

Role of cell physiology and population effects in resistance

The growth rate of a bacterium depends on the nutrient environment and is a key physiological parameter that can strongly affect its sensitivity to a wide range of antibiotics [15,24$^{\bullet\bullet}$,25,26]. A recent experimental-theoretical study focused on ribosome-binding antibiotics, and showed that a lower growth rate (achieved by different growth media) increases the tolerated antibiotic concentration while for others, the opposite effect occurs [24$^{\bullet\bullet}$]. A mathematical model based on bacterial 'growth laws' [27], which take into account how the ribosome concentration in the cell depends on growth rate, showed that the ribosome-binding kinetics of the drug can explain this effect: slow-growing cells are more resistant to reversibly binding drugs, whereas fast-growing cells are more resistant to irreversibly binding drugs [24$^{\bullet\bullet}$]. These results would have been hard to intuit without using a rigorous theoretical approach and highlight that apart from specific molecular mechanisms, global cell physiology and growth rate are important determinants of antibiotic resistance levels.

Glossary

MIC: Minimal inhibitory concentration. The lowest concentration of an antibiotic that completely inhibits growth of a clonal culture.

TEM-1 β-lactamase: An enzyme produced by bacteria that cleaves and deactivates β-lactam antibiotics.

DFE: Distribution of fitness effects. The probability distribution that represents the changes in fitness caused by single-step mutations originating from a common genotype. It depends on the ancestral genotype and on the environment.

Epistasis: The phenomenon that the effect of a mutation depends on the genetic background it occurs in.

Discrete fitness landscape: A graph in which the vertices are genotypes, each with an assigned fitness value. Two genotypes are connected by an edge if they are a single mutational event apart.

Global cell physiology can even explain how a clonal population diversifies into growing and non-growing cells in the presence of antibiotics. The expression of many genes increases with increasing growth rate [28]; this effect alone can lead to bistable population dynamics [29]. Specifically, it was shown that in an *Escherichia coli* strain that expresses the cat1 enzyme, which inactivates the antibiotic chloramphenicol (Table 1), a positive feedback loop occurs where a decrease in growth rate due to addition of more chloramphenicol decreases expression of the resistance-conferring enzyme, thus slowing growth even further [30]. Theory shows that such a positive feedback loop can lead to bistability, that is, coexistence of growing and non-growing cells at the same drug concentration; this striking effect was confirmed in single cell experiments [30]. Growth bistability is likely a more general phenomenon [31] that occurs for other resistance mechanisms and highlights that the response of a population of clonal bacteria to antibiotics is not simply given by many identical copies of the same cell.

Population effects are also important when resistance is due to extracellular antibiotic degradation. Here, the antibiotic concentration in the medium strongly depends on the cell density, since higher densities lead to faster antibiotic degradation. The inoculum size of the culture thus affects the growth of all cells and, ultimately, the measured resistance level. This effect has been described in mathematical models and experimentally validated using the beta-lactamase enzyme which degrades beta-lactam antibiotics (including amoxicillin, ampicillin, and cefotaxime) [32,33]. Such effects generally occur whenever a resistant subpopulation degrades or modifies the antibiotic so that the entire population can benefit from it. It will be interesting to further investigate the causes and consequences of these effects which also occur for other antibiotics [34].

Studying antibiotic resistance using experimental evolution

Beyond characterizing existing resistance mechanisms, it is a fundamental question how *de novo* resistance evolves. Understanding this can ultimately lead to strategies for inhibiting resistance evolution. Recent years have seen a plethora of novel techniques for investigating antibiotic resistance evolution in the laboratory and for systematically addressing its reproducibility, speed, molecular origins, and constraints.

Resistance often evolves so fast that it can be studied in the laboratory but it is still challenging to obtain quantitative and reproducible results. Serial transfer of microbial cultures is a common experimental evolution protocol [35] that is also useful for studying resistance evolution [22,36,37]. In this protocol, bacterial cultures grow in flasks or on microtiter plates and are diluted into fresh medium by a fixed factor at regular time intervals (*e.g.*, every 24 hours). These experiments can be continued virtually indefinitely: Richard Lenski's seminal long-term evolution experiment [35] has exceeded a staggering 60 thousand generations in 28 years and is still ongoing. Because of the relative simplicity of this protocol, it is feasible to run hundreds of evolution experiments in parallel. Together with increasingly inexpensive whole genome sequencing techniques [38], this opens the door for a statistical investigation of the intrinsically stochastic evolutionary dynamics and for identifying general principles governing microbial evolution [39–42]. A drawback of serial transfer protocols is their inability to keep key parameters that affect the evolutionary process well-controlled: the population size fluctuates considerably and cultures differ in their growth rates and in the time they spend in stationary phase. This complicates the quantitative investigation of the evolutionary process and its comparison among different cultures. Furthermore, it is not straightforward how the antibiotic concentration should be chosen in such experiments to gain maximum insight into the process of resistance evolution: if it is too low, there is virtually no selection for resistance; if it is too high, cells cannot grow at all, preventing them from evolving at a significant rate.

Recently developed techniques in which bacteria are exposed to increasing antibiotic concentrations solve this problem. Theoretical work suggested that temporal or spatial selection gradients can facilitate the sequential emergence and fixation of multiple resistance mutations leading to increasingly higher resistance levels [43,44]. Consequently, advanced protocols that gradually increase antibiotic concentrations in time or space have been

**Table 1**

Glossary of antibiotics and their targets

Antibiotic	Target
Chloramphenicol	50S ribosomal subunit
Tetracycline	30S ribosomal subunit
Amoxicillin	Cell wall synthesis
Trimethoprim	Folate synthesis (DHFR)
Ciprofloxacin	DNA replication (DNA gyrase)

developed [45••,46–48]. A notable example is the 'morbidostat': this feedback-controlled device keeps cultures growing in exponential phase and automatically increases the antibiotic concentration during the experiment such that they keep growing at a pre-defined rate despite their increasing resistance. In this way, strong selection pressure for resistance is constantly maintained. For some antibiotics, this protocol enabled the highly reproducible evolution of a ~1000-fold resistance increase in just a few weeks [47].

Spatial antibiotic gradients may enable even faster resistance evolution. A striking example is given by a recently developed microfluidics device: a concentration gradient of the fluoroquinolone antibiotic ciprofloxacin (Table 1) was maintained across this ~2–3 cm hexagonal device that consists of over 100 micro-compartments that are connected, allowing cells to move between different concentrations. From an initial population size of 10^6 cells, the authors observed a surprisingly strong, over 200-fold resistance increase that resulted from multiple reproducible mutations that had occurred as early as 10 hours after inoculation [48]. A recent study sized

the assay oppositely and followed evolution on a huge, meter-scale agar plate (the 'MEGA plate') [45••]. In contrast to small agar plates where rapid diffusion quickly destroys spatial drug concentration gradients, they remain relatively stable on this larger plate. Further, the size of the plate allows for large bacterial population sizes that should accelerate the occurrence of resistance mutations. Fast resistance evolution reaching extremely high levels within weeks was observed for different antibiotics. Changing the slope of the concentration gradient revealed that smaller steps in drug resistance enable the multi-step evolution of high resistance levels that are practically impossible to reach in a single mutation step [45••]. A fascinating aspect of this experiment is that the front of bacteria that grows across the plate can be viewed as a living Muller diagram that directly visualizes the evolutionary record and the key role of stochastic events in this process (Figure 1a,b): some of the most highly resistant lineages ultimately stalled in this assay because they emerged in an unfavorable location too far away from the growth front [45••], illustrating the stochasticity of the process. Together, these results highlight the great potential of new assays with well-defined spatial

Figure 1
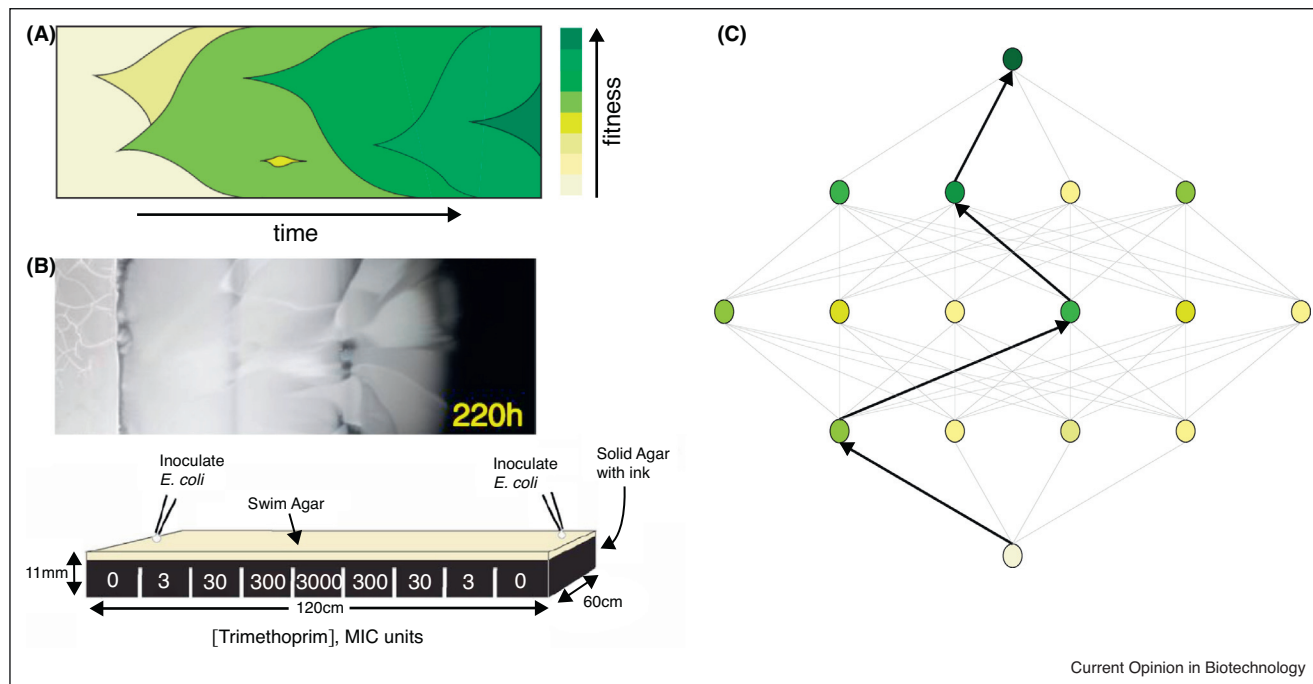
Schematic representation and direct visualization of evolving populations on fitness or antibiotic landscapes.
(a) A Muller plot showing the relative abundance of genotypes in an evolving population. Each colored region represents a subpopulation with a different fitness; darker green indicates higher fitness. **(b)** Top: a photograph of the MEGA-plate showing growth of *E. coli* over bands of increasing trimethoprim concentration (left to right). Snapshot after 220 hours of growth. Bottom: a schematic of the MEGA-plate experimental setup. From Ref. [45••], reprinted with permission from AAAS. **(c)** The same evolving population as in a, represented on a discrete fitness landscape of four mutations: circles represent all possible genotypes of four loci, each with two alleles. Two points are connected by an edge if the two genotypes are one mutational event apart. The arrows show the only path that has monotonically increasing fitness from the least fit to the fittest genotype.

drug landscapes as tools for investigating resistance evolution.

Apart from experiments where the antibiotic concentration increases monotonously, evolution has been studied under different temporal sequences of antibiotic exposure. In a recent experiment, *E. coli* cultures were repeatedly exposed to a high concentration of ampicillin for a fixed time, followed by complete removal of the drug and growth in its absence. In this assay, the bacteria did not evolve resistance at all but instead genetically tuned their lag times to match the duration of the antibiotic exposure [49••]—a stunning observation and an effective survival strategy as the antibiotic used can only kill growing cells. The effect of various temporal exposure protocols on resistance dynamics was also studied at the single-cell level. A recent study used a synthetic stochastic switch controlling tetracycline resistance and observed the effect of antibiotic pulse length on the probability of selective sweeps in a microfluidic device; an intermediate regime in which sweeps are unlikely was identified [50]. Overall, these studies provide powerful tools for observing resistance evolution at different levels; however, mathematical models are needed to interpret the data and extrapolate to predictions beyond the laboratory.

Quantitative understanding of resistance mutations and their genetic interactions

The key ingredients entering theoretical descriptions of evolution are mutation and selection. While mutation rates can be estimated [51], the fate of mutations in the face of selection is determined by their effects on survival and growth (fitness) of the organism in the current environment. In theory, the probability that a new mutation has a certain fitness effect is determined by the so-called distribution of fitness effects (DFE) [52]. In practice, the shape of this distribution is hard to measure and has remained elusive. Approximations of the DFE for specific environments can be obtained by direct competition with the ancestor [42,53], or by comparing growth rates [54••] or survival in high drug doses [55] for a large number of mutants. The shape of the DFE is crucial for the evolutionary dynamics. For example, it is a classical result that, for two DFEs with the same mean fitness effect but different variances, the one with the greater variance provides a greater probability for the occurrence of highly beneficial mutations and thus speeds up evolution. Recent studies have measured DFEs relevant for the specific case of antibiotic resistance evolution and revealed general relations that partly explain the shape of this distribution [46–48].

The width of the DFE in the presence of antibiotics was shown to depend strongly on the dose–response characteristics of the drug. The DFEs for eight antibiotics spanning diverse modes of action were approximated by measuring the growth rates under those antibiotics for all ∼4000 strains of the *E. coli* gene deletion library [54••,56]. Interestingly, the widths of the distributions vary drastically across antibiotics. These differences are largely explained by the shape of their dose response curves: when the growth rate is sensitive to small differences in the concentration of a particular antibiotic, the corresponding DFE is wide. Conversely, for antibiotics where the growth rate is robust to such small dose differences, the corresponding DFE is narrow (Figure 2) and can even become narrower than in the absence of drug. A population genetics model predicted that the rate of resistance evolution and the diversity of evolutionary paths should increase for antibiotics with greater DFE width when compared to antibiotics with similar dose–response characteristics. These predictions were confirmed in evolution experiments using the morbidostat [54••]. These results highlight the potential of identifying key factors that determine the shape of the DFE for different antibiotics and bacteria; identifying such factors can enable increasingly accurate predictions of resistance evolution.

DFEs of mutations restricted to specific resistance genes can be measured more comprehensively and have revealed surprising features. A notable example is the DFE of the TEM-1 β-lactamase that was tackled in several recent efforts. First, beneficial mutations were detected by screening a randomly mutagenized library for TEM-1 variants that convey resistance to cefotaxime, and their resistance levels were quantified [57]. Second, the amoxicillin MIC was measured for 64% of all possible amino-acid substitutions in the TEM-1 enzyme; their effects were partially explained by amino acid properties and calculated protein stability changes [55]. Both studies consistently found that a few mutations have a much greater effect on fitness than predicted from an often assumed exponential DFE. This result suggests that evolution may be more predictable than expected—at least for resistance enzymes. Finally, a high-resolution map of the fitness effects for over 98% of all possible point mutations in the TEM-1 gene was assembled; this map suggested that the genetic code biases mutations toward beneficial effects [58]. The TEM-1 β-lactamase became a model system for the detailed understanding of DFEs in the context of antibiotic resistance; results from this system suggest that amino-acid properties and protein stability can help to predict the effects of many mutations.

The DFE generally depends on the genetic background; it can thus change as soon as the first mutation has occurred. The phenomenon where the effect of mutations depends on the presence of other mutations is termed 'epistasis' [59]. Measuring the extent of epistasis is important for evolutionary predictions because prevalent epistasis often leads to multiple fitness peaks and can prevent a population from reaching the global fitness maximum [60•]; in particular, this is the case for

Figure 2
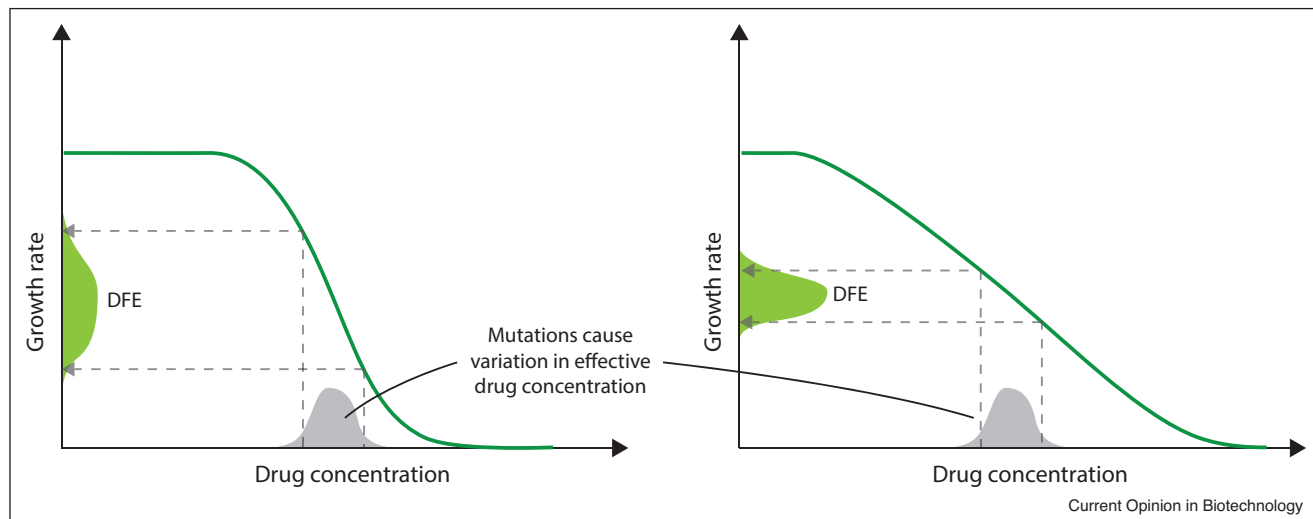
Dose–response characteristics of antibiotics shape the distribution of fitness effects.
Schematics of two different dose response curves: the left curve is steep, that is, the growth rate is sensitive to small changes in drug concentration; the right curve is shallow. Mutations cause shifts in the effective drug concentration a bacterium experiences; the typical magnitude of these shifts is surprisingly similar for diverse antibiotics [54••]. The distribution of effective drug concentrations resulting from many different mutations is shown in gray. These mutations produce distributions of growth rates (fitness) that are wide for the steep dose–response curve (left) and narrow for the shallow dose–response curve (right).

'reciprocal sign epistasis' where the fitness effect of a mutation changes from positive to negative, depending on the background [61]. Epistasis can be analyzed using discrete fitness landscapes which are a powerful metaphor for assessing the constraints and predictability of mutational paths in evolution experiments [62•,63]. A discrete fitness landscape is a graph in which the vertices are genotypes, and two genotypes are connected by an edge if they are a single mutational event apart. The landscape is completed by assigning a fitness value to all genotypes (Figure 1c). Paths on the landscape are accessible if they represent sequences of genotypes with monotonically increasing fitness, that is, all mutations along the path are beneficial. If only few of the possible paths are accessible, evolutionary trajectories become more constrained and predictable. Due to the astronomically large number of possible genotypes, it is not feasible to measure the fitness effects of all mutations and their combinations experimentally, even for short sequences. Therefore, recent studies have focused on full landscape reconstructions of just a few mutations relevant for drug resistance [62•,63,64] and proposed biophysical models to predict epistatic interactions from protein structure and function [65,66•].

The enzyme dihydrofolate reductase (DHFR) has served as a key model for describing higher-order epistasis and biophysical constraints of fitness landscapes. DHFR is the target of trimethoprim (Table 1) and can cause resistance to this antibiotic via a few point mutations. A recent study reconstructed a fitness landscape of seven known resistance mutations in DHFR [62•]. It showed that when the possibility of the same locus mutating more than once is taken into account, prevalent epistasis may increase the accessibility of all peaks on a landscape, decreasing the chance of being 'trapped' at a suboptimal fitness [62•]. A later study measured the effects of three resistance mutations in DHFR and their combinations on enzyme efficiency, stability, and ability to bind trimethoprim [66•]. It discovered a trade-off between affinity to trimethoprim, enzyme efficiency, and stability which shapes the epistatic interactions in the fitness landscape. Further, the activity of protein chaperones strongly affected the shape of the fitness landscape by changing the stability of the enzyme [66•]. Together, these results underline the importance of genetic interactions both within the same gene and across different cellular mechanisms for predicting evolution.

A broader investigation of interactions between drug resistance and other cellular functions, including seemingly unrelated ones, can uncover potentiators of resistance evolution, that is, genes that accelerate this process [67]. Notable examples are mechanisms that increase genetic variability by increasing the mutation rate in response to an antibiotic challenge (stress-induced mutagenesis). In particular, this can happen by upregulation of the mutation-inducing SOS response [68–70], induction of mutagenic oxidative damage [71,72], or by regulated DNA uptake from the environment [73]. Mechanisms

that affect evolvability are an interesting potential target for new drugs that could be combined with established antibiotics to hamper spontaneous resistance evolution—an idea that has triggered efforts to develop SOS response inhibitors [68,74,75].

In addition to potentiation through changes in mutation rate, it will be interesting to identify mutations in other cellular functions that may increase the rate of resistance evolution via epistatic interactions. A conceptually related phenomenon occurred in Richard Lenski's long term evolution experiment where certain potentiating mutations were required before the ability to metabolize citrate could evolve [76,77]. At the heart of this phenomenon is a particular substitution with minor effects on fitness that allows for a secondary mutation to become beneficial. Similar genetic interactions between resistance mutations and genetic background can occur [78], suggesting that the evolvability of antibiotic resistance can be strongly affected by the presence of mutations in diverse cellular pathways. A systematic identification of mutations that produce significant changes in resistance development would greatly enhance our understanding of the complex interplay between drug resistance and other cellular functions. If the effect of these mutations can be chemically mimicked, this research could lead to the discovery of new adjuvants to antibiotics that slow down resistance evolution.

Conclusions

Over the last decade, the field has made considerable progress in understanding antibiotic resistance evolution, at least in well-controlled laboratory settings. Bacterial growth laws have helped to elucidate the interplay between cell physiology, antibiotic action, and resistance and made accurate quantitative predictions of surprising antibiotic effects. The success of studies thus far [24••,30] holds promise that a quantitative characterization of bacterial physiology will also lead to an improved understanding of resistance mechanisms for antibiotics with other targets than the ribosome.

New technology for evolution experiments together with improved mapping of mutational fitness effects and epistatic interactions will soon allow us to statistically test predictions for evolution in various simple and structured environments. A key challenge is to scale recently developed, precisely controlled lab evolution protocols [47] to higher throughput so that many antibiotics and various strains can be tested in parallel at high replication. This technology would enable a systematic investigation of mutations and other perturbations that affect resistance evolution. It would further provide a deeper understanding of the general principles of resistance evolution and enable predictions of the differences in the propensity for resistance evolution among bacteria and antibiotics. Beyond such well-controlled experiments, a great challenge is to develop technology enabling experiments that mimic the physiological environment as it occurs in an infection. Apart from the host immune system and physical properties of the host environment, the presence of other microbes at the infection site can affect the success of antibiotic treatments targeted at a single pathogen. In the long run, it will be crucial to translate the advances on antibiotic resistance evolution into specific intervention strategies that are effective against pathogenic bacteria in an infected host but, unlike current treatments, keep resistance in check.

Acknowledgements

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- •• of outstanding interest

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