Evolutionary mechanisms shaping the maintenance of antibiotic resistance

Paulo Durão[1], Roberto Balbontín[1], Isabel Gordo[1*]

[1]Instituto Gulbenkian de Ciência, Oeiras, Portugal

*Correspondence: igordo@igc.gulbenkian.pt (I.Gordo)

Keywords: Antibiotic, evolution, fitness costs, compensation, epistasis, multidrug resistance;

Abstract

Antibiotics target essential cellular functions but bacteria can become resistant by acquiring either exogenous resistance genes or chromosomal mutations. Resistance mutations typically occur in genes encoding essential functions, which causes resistance mutations to be generally detrimental in the absence of drugs. However, bacteria can reduce this handicap by acquiring additional mutations, known as compensatory mutations. Genetic interactions (epistasis) either with the background or between resistances (in multi-resistant bacteria) dramatically affect the fitness cost of antibiotic resistance and its compensation, therefore shaping dissemination of antibiotic resistance mutations. This review summarizes current knowledge on the evolutionary mechanisms influencing maintenance of resistance mediated by chromosomal mutations, focusing on their fitness cost, compensatory evolution and epistasis and the effect of the environment on these processes.

The threat of bacterial antibiotic resistance

The introduction of antibiotics represented one of the most important medical interventions in the history of global health resulting in a dramatic reduction in human morbidity and mortality caused by bacterial infections. However, the intensive use of antibiotics has accelerated the dissemination of bacteria that evolved to endure these drugs through the acquisition of genes or chromosomal mutations that confer resistance [1,2]. Antibiotic resistance (AR) is widespread in clinical [3,4] and environmental settings [5,6], providing a reservoir that can further spread by horizontal gene transfer. AR is a serious and growing challenge in the treatment of infectious disease (http://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/), with single and multidrug resistant clones of major pathogens circulating at frequencies above those expected by a balance between the stochastic emergence of resistance by mutation and a purge of deleterious mutations (in the absence of antibiotic) by natural selection [7]. The dissemination of multidrug resistant bacteria is unfortunate because these clones are harder to treat and those harbouring mobile resistance elements (MREs) are able to spread resistance more quickly. Antibiotic resistant infections world-wide are estimated to potentially cause millions of deaths by 2050 (https://amr-review.org/) and already inflict a major economic toll [8]. Beyond complex demographic processes, the emergence and dissemination of AR in bacterial populations depends on key evolutionary parameters, such as (i) the rate at which bacteria acquire resistance, (ii) the selective pressures for and against resistant bacteria (see Glossary), and (iii) the rate and effects of mutations compensating for potential costs of resistance (see Glossary) [9]. These factors have been shown to be influenced by both the genetic background and the environment in which resistant bacteria grow [10], underlining the necessity of experimental studies and quantitative analysis of these rates and processes *in vivo*, e.g. in ecological contexts related to infections, and in nature, e.g. in microcosms mimicking natural habitats. Such studies are vital to preserve the effectiveness of antibiotics and reduce the frequency of resistance in bacterial populations. Here we summarize up-to-date knowledge on the evolutionary mechanisms influencing maintenance of resistance mediated by chromosomal mutations. In particular, we will focus on fitness cost of AR, compensatory evolution, epistasis and environmental effects on these evolutionary mechanisms.

Emergence of bacterial antibiotic resistance

The rate of appearance of antibiotic-resistant bacteria is determined by the combined rates of *de novo* mutation (U) and horizontal gene transfer (HGT) of mobile genetic elements carrying resistance (MRE, see **Box 1**). While acquisition of new DNA requires specific ecological contexts (i.e.: the presence of donor bacteria), adaptive mutations (potentially including resistance mutations) are continuously generated at rates that can be as high as ~10^{-5} per cell per generation [11–13]. Furthermore, mutations leading to genomic rearrangements (insertions, deletions, duplications, inversions) occur at an even higher rate (10^{-3}-10^{-5} per cell per generation) which can accelerate the rate of acquiring AR [14,15]. The rate of the emergence of AR mutants is affected by physiology, genetics, antibiotic-bacterium interactions (e.g.: antibiotic itself can affect mutation rate [16] or different resistance mutations can be selected at different antibiotic doses [17]), and the current and past environment to which bacteria have been exposed (e.g.: bacteria grown at high temperature can acquire *rpoB* mutations conferring rifampicin resistance [18]), together with the physical structure of the selective medium [12].

It is important to note that antibiotic stress itself can impact the general value of U. Indeed, a growing body of evidence suggests that sub lethal concentrations of several antibiotics can boost resistance emergence via increasing the rate and frequency of HGT, recombination, and mutagenesis [19]. Furthermore, bacteria can acquire mutations that increase their genome-wide U typically 10 to 1000-fold – known as “mutators”. In fact, it has been long known that recurrent pressure of antibiotics selects for mutator clones due to their increased ability to produce the rare mutations that can rescue bacterial populations from such high selective pressures [20]. Mutators are also known to exhibit increased ability for recombination [21]. In samples of natural isolates of *Escherichia coli*, clones with intermediate mutator phenotypes have been found to carry significantly more AR mutations [22].

Hence, bacteria have an enormous potential for adaptation with access to a large supply of mutations and exogenous genetic material that could explain why AR evolves remarkably quickly both in laboratory and clinical environments [23,24].

However, in the context of infection, bacterial population sizes within hosts are high enough (above 10^{10} in certain contexts) to already include pre-existing resistant mutants [9]. In such a large bacterial population, and considering a base-substitution mutation rate of around 10^{-}

^{9}-10^{-10} per nucleotide site per generation [25], it is possible that all viable mutations, including resistance mutations, would already exist in the population. Thus, the current estimates of mutation rates and the large population sizes suggest that U may have a limited influence on the emergence of resistances. On the other hand, population genetic theory has shown that i) if a population is facing antibiotic pressure once a resistance mutation arises, its chances of not getting lost and spreading depend on its beneficial fitness effect (its selection coefficient); ii) if a population does not experience antibiotic pressure, the resistance mutation is expected to attain a frequency reflecting the balance between the rate of production of the mutants (proportional to U) and the rate of elimination by natural selection (the deleterious fitness effect resulting from a cost of resistance) or by genetic drift (random changes in the frequency of resistant mutants in a population). In light of the unavoidable escape of recurrent mutations associated with cell division, restriction of resistance relies on the power of purifying selection acting on the costs that resistance mutations might cause (**Figure 1**) [26,27].

Fitness costs associated with antibiotic resistance

From *in vitro* studies, the acquisition of resistance is often associated with fitness costs in the absence of antibiotics [1,4,28]. Deleterious effects are thought to originate either from the cost of maintaining resistance carrying plasmids (see **Box 1**) [29–36] or from the pleiotropic effect of chromosomal resistance mutations [1,4,37]. In the latter case, costs are often associated with the fact that resistance mutations map onto genes encoding essential cellular functions (targeted by antibiotics), such as transcription, translation or cell wall biogenesis.

The existence of a fitness cost caused by AR predicts that the fitter susceptible strain should outcompete the resistant strains over time (**Figure 1**) [28]. This is in agreement with the observed decrease of antimicrobial resistance in clinical settings when the use of certain antimicrobials is halted [38–41]. However, costs are not always found to occur [28]. For example mutations causing streptomycin and/or rifampicin resistance could confer survival benefits to bacteria engulfed by macrophages [42,43]; mutations conferring rifampicin resistance could spread to high frequencies in bacterial populations growing under limited resources [18,44]; mutations conferring carbapenem resistance were found to confer a competitive fitness advantage to *Pseudomonas aeruginosa* colonizing the mouse intestine

and disseminating to the spleen [45]; carbapenem and fosfomycin resistance mutants can have increased virulence in a murine pneumonia model [46]; and mutations conferring vancomycin resistance can be selected for as a result of competition between diversified genotypes of *Staphylococcus aureus* spontaneously generated from a common ancestral strain [47].

The examples above also underline the strong influence of the environmental conditions on the fitness cost of resistances [37,48,49]. However, based on the studies that have been performed, fitness measurements made in the laboratory settings appear to have clinical relevance since they agree with epidemiological studies of the prevalence of resistance alleles in clinical isolates [50–52].

Benefits and costs in the presence of antibiotics

While fitness effects of resistance mutations show a strong genotype-by-environment interaction [49] in the absence of antibiotics, their benefits are less dependent on the environment complexity if high antibiotic pressure is applied. This permits the use of experimental evolution in the lab to anticipate the spectrum of beneficial mutations causing resistance to high antibiotic doses. Indeed many of the resistance mutations in clinical isolates can be evolved in the lab, under the appropriate selective pressure [53].

The acquired level of resistance to the antibiotic is experimentally measured through the minimal inhibitory concentration (MIC). The level of resistance can vary extensively depending on the resistance mechanism and the conditions under which resistance is measured. For instance, if the resistance mechanism affects monotonically the growth with the drug concentration, then the relative fitness of an antibiotic-resistant bacterium might vary extensively depending on antibiotic concentration [9,54]. While it is clear that the high concentrations of antibiotics used therapeutically can select for resistant mutants, it has been shown both in *E. coli* and *Salmonella enterica* that concentrations of tetracyclines, quinolones, and aminoglycosides hundreds-fold below the MIC of susceptible bacteria can select for resistant bacteria [3,55]. Importantly, it is not currently known if measurements of resistance levels in the laboratory, typically performed in conditions far from natural, can be extrapolated to resistance levels of bacteria in a host. In a host, inter-species ecological interactions are likely to occur that are inexistent in most *in vitro* studies, some of which could buffer the antibiotic pressure experienced by a particular bacteria [56,57].

An interesting environmental effect mediated by the presence of antibiotics on the fitness of resistant strains relates to the potential biological activities in degrading antibiotics. For instance, certain products of the physicochemical degradation of tetracycline are more harmful for resistant than for sensitive *E. coli*, causing the competitive advantage conferred by the resistance to eventually reverse and become disadvantageous [58].

Troublingly, certain mechanisms confer resistance to antibiotics at unknown fitness costs. For instance, a set of mutations in genes encoding ribosomal components in *Mycobacterium smegmatis* confer resistance to diverse antibiotics not related structurally or mechanistically, by causing extensive transcriptomic and proteomic changes, affecting proteins known to impact AR [59]. Furthermore, bacterial populations can collectively survive antibiotic treatments lethal to individual cells via diverse mechanisms, such as production of resistance enzymes, bistable growth inhibition mediated by antibiotic titration, swarming or interactions between different bacterial subpopulations. These strategies allow bacterial populations to survive upon antibiotic treatment and provide a time window for the acquisition of genetic resistance [60].

Compensation of the fitness costs

Despite the importance of the fitness costs in predicting the dissemination of AR mutations, there are additional factors that significantly affect the evolutionary path of AR. The rapid acquisition of compensatory mutations by resistant clones is key to prevent them from being outcompeted by sensitive bacteria, as widely described for the cost of single resistance both in clinical [61,62] and laboratory conditions [63–67]. Compensation can occur either by losing the original resistance mutation which is causing the fitness decrease – a process known as reversion (**Box 2**, recently discussed in [68]) - or by acquiring additional mutations which counteract the cost. Compensatory mutations generally affect genes encoding proteins involved in cellular machinery functionally related to those affected by the original mutation [69].

The dynamics of compensatory adaptation depends on population size, bottlenecks [63], mutation rate [70], and the distribution of fitness effects of compensatory mutations, which depends on the genetic background due to genetic interactions [71,72]. For instance, in the simplest case of a single resistance mutation, it has been shown that compensation is typically faster when the fitness cost of the resistance mutation is higher, leading to the

prediction that clones carrying more costly resistance mutations have higher adaptive potential. This faster adaptation is likely driven by the acquisition of compensatory mutations with larger effects on these backgrounds [73]. In the more complex case, where a population carries genetic variation for resistance mutations, the different clones will have different distributions of compensatory mutations and high competition between clones with different fitness - clonal interference – may result in the maintenance of costly resistance alleles over long periods of time. For example, a study using experimental evolution with resistant *E. coli* clones observed coexistence between costly rifampicin and less costly streptomycin resistance mutations during hundreds of generations [71]. This type of study exposes the complexity of the fitness landscape and the evolutionary dynamics, which impacts predictions about extinction of high cost resistances.

Even though compensation of resistant bacteria is often studied in the absence of antibiotic [65–67,70], it can also occur in the presence of antibiotics. For instance, mutations that decreased both the cost of resistance to fluoroquinolone and the susceptibility to the antibiotic have been described [74,75]. The few studies that have compared bacterial compensation in the absence versus presence of antimicrobial selection pressure [64,76] indicate that both the targets of compensation in the presence of antibiotic and their fitness effects can be different from the ones in the absence of the drug. For instance, mupirocin resistant mutants, carrying compensatory mutations acquired in absence of the drug, have increased fitness only in this environment and not when the antibiotic is present [76].

The effects of the presence or absence of antibiotics on compensatory evolution of resistant bacteria become particularly relevant in light of the current discussion on the appropriate duration of antibiotic treatments [77]. Although for certain infections there is strong evidence on what is the optimal duration of an antibiotic course, this is unknown for many other infections [78]. In case of long treatments (**Fig. 1**B top panel), resistant mutants are able to reach large population sizes, which favours compensation during the antibiotic treatment. Thus, compensation in presence of antibiotic becomes more significant, as the effects of compensatory mutations acquired during the antibiotic treatment on bacterial fitness in an antibiotic-free environment will likely determine whether compensated resistant bacteria can be outcompeted or not. Conversely, in case of short treatments (**Fig. 1**B bottom panel), resistant mutants are unlikely to take over the entire population, making compensation during treatment much more difficult. In these cases, compensatory evolution

in absence of antibiotics constitutes a better framework for predicting the evolutionary fate of resistant bacteria. Efforts to elucidate the optimal duration of antibiotic treatments for each infection are therefore essential to determine the most relevant environment to study compensatory evolution and, subsequently, elaborate predictions on the evolutionary trajectories of resistant pathogens [9].

Another example of environmental effects on compensation with relevant clinical implications was the observed selection of different compensatory mutations depending on whether the resistant bacteria evolved in mice or in laboratory conditions, indicating that compensatory evolution can take different trajectories within and outside a host [67]. Indeed, the clinical and epidemiological importance of compensation remains poorly understood [4,79–81].

Epistatic effects on antibiotic resistance

Epistasis occurs when the effect of a mutation depends on the genetic background where it arises. It is has been shown that the same AR mutation can have different effects if it occurs in different genomes [82–84]. For example, strains harbouring identical rifampicin resistance mutations but belonging to different lineages of *Mycobacterium tuberculosis* showed different levels of fitness cost [79]. Likewise, the available data suggests that the bacterial genetic background can also influence the fitness of bacteria with MRE [36,85,86]. Epistasis can have profound implications for the spread of bacterial AR [48,87–89]. In the simplest case, epistasis can be quantified between two loci - pair-wise epistasis- and it can be *positive* or *negative* (see **Figure 2**A for details).

Positive (negative) epistasis occurs when the fitness of a clone carrying mutations at the two loci is higher (lower) than expected given the effects in fitness of each of the single mutants. Furthermore, an important form of interaction – *sign epistasis* - can occur if the sign of the effect changes from deleterious to beneficial (or vice-versa) in the double mutant [90]. Non-reciprocal sign epistasis occurs when the double mutant fitness is higher (or lower) than one of the single mutants, whereas reciprocal sign epistasis occurs when the double mutant fitness is higher (or lower) than both single mutants (**Figure 2**A). The strength and type of epistasis is also known to depend on the environmental context, as expected given that the fitness effects of resistance differ with the growth media [49].

Epistasis strongly affects the dissemination of AR because it can greatly influence the dynamics and repeatability of evolution at numerous stages [88,91–95]. For instance, during a constant antibiotic treatment a phenomenon called *diminishing returns epistasis* can occur, where the beneficial effect of the resistance mutations decreases as they sequentially accumulate, limiting the subsequent evolution [84].

Epistasis has a decisive role during compensation of costly AR mutations in the absence of drugs. Most compensatory mutations are deleterious or neutral in the sensitive background, but advantageous in the resistant background [72]. As a consequence, the persistence of resistance mutations upon compensation is promoted because reversions will strongly be selected against. A bacterial population enriched with resistant mutants carrying compensatory mutations can readily acquire a second resistance, either by accumulating chromosomal mutations selected for in the presence of a new antibiotic, and/or by acquiring plasmid-borne resistant elements (**Box 1**), leading to multidrug resistant strains [80,96].

Importantly in the context of multiple-resistance, different resistance mutations can also interact epistatically. Studies in *E. coli*, *P. aeruginosa*, *M. tuberculosis*, *Salmonella enterica* and *Streptococcus pneumoniae* found many instances of positive epistasis, with the observed cumulative fitness cost of carrying multiple drug resistance-conferring mutations below the expected sum of the fitness costs associated with each individual mutation [48,87,97–99]. Positive epistasis between chromosomal resistance mutations and MRE or between different MRE has also been observed (**Box 1**). Pervasive positive epistasis was found not only between costly resistance mutations but also when combining costless rifampicin resistance alleles with costly streptomycin resistance alleles [48]. Moreover, double resistant clones were also shown to exhibit sign epistasis [87,90], with the implication that in the absence of antibiotics the acquisition of further resistance mutations (or eventually plasmids) can increase the fitness of an initially single resistant strain, resulting in reduced probability of reverting resistance by halting drug use.

Fortunately, although not as commonly as desired, examples of pairs of resistance mutations which interact negatively have also been found [87]. Knowledge of these negative epistatic interactions between resistance mutations is important and can be clinically explored to slow down the evolution of multi-resistance by using specific combinations of antibiotics. If, for a given pair of drugs, negative epistasis is expected to dominate the landscape of potential emerging resistance mutations, then the few double resistant genotypes that

would survive the treatment would have highly reduced fitness, and be outcompeted by single resistant and/or susceptible genotypes once the antibiotic treatment is completed.

Importantly, resistance to one drug might also increase susceptibility to another drug – a phenomenon called collateral sensitivity [100] – which constitutes another relevant interaction that can be used to combat resistant strains.

Notably, compensatory evolution of multi-resistant strains can also be affected by epistasis. Although this topic remains poorly explored, the common observation of epistasis between resistance mutations implies that compensation of multiple-resistance bacteria can significantly differ from that of single resistant strains (**Figure 2**B). In the case of positive epistasis one could expect that the process of compensation would entail less compensatory targets than those involved in the compensation for costs of each single resistance. This should be especially strong under sign epistasis, where multi-resistant clones have higher fitness than some of the single resistant clones. On the contrary, negative epistasis should result in a higher number of compensatory targets, as mutations specifically compensating for the negative epistasis could be expected. A recent study [72] showed that this can indeed occur. By following the compensatory process of a streptomycin and rifampicin double-resistant *E. coli* and comparing it with that of single-resistant clones, the study unveiled mutations in gene targets that only compensate for double resistance, e.g a specific amino acid change in *rpoC* and a mutation causing increased expression of *nusG*. These mutations were neutral or deleterious in sensitive or single resistant backgrounds, demonstrating their compensatory nature solely under double-resistance. The study also showed that the compensatory effect of the mutations disappeared in an environment where the epistatic interaction between resistance alleles was absent, consistent with the hypothesis that these mutations were specifically compensating for the epistatic interaction between the ARs [72].

The detection of compensatory targets for epistasis can lead to the identification of proteins involved in multiple essential processes. These proteins are potential targets for the development of new antimicrobials, since their functional inhibition could strongly affect bacterial fitness, furthermore limiting the rise of resistance mutations because these would be particularly deleterious in these conditions.

Epistasis can also occur at the intragenic level. There is plentiful evidence for sign epistasis during the evolution in β-lactamases towards high levels of AR [88,91,101,102]. Remarkably, sign epistasis was shown to limit the number of evolutionary paths available to evolve

increased resistance. For instance, during the evolution of classical β-lactamases into extended-spectrum β-lactamases (ESBL), pervasive sign epistasis between mutations was observed, where many mutations, individually leading to increased ability to degrade cephalosporins, showed decreased MIC when combined [88]. In the system studied only 18 out of the 120 possible evolutionary pathways continuously increased the MIC.

A likely reason for such frequent epistasis is that mutations are often pleiotropic, simultaneously affecting multiple phenotypes [91]. Pleiotropy is a key assumption in classical models of adaptation to novel environments such as Fisher's geometric model (FGM, see **Box 3**), which describes the relationship between multiple phenotypic traits and fitness, and predicts complex patterns of epistasis [103–105].

A common form of pleiotropy within proteins is the simultaneous effects of mutations on enzyme activity and stability [101,106,107]. For instance, on the β-lactamase TEM-1, mutations which increased activity against cephalosporin antibiotics lost thermodynamic stability. However, a second mutation which is neutral or deleterious by itself stabilizes the proteins carrying an activity-increasing mutation, another example of sign epistasis [106]. Interestingly, it has also been shown that the deleterious effect of a fraction of the destabilizing mutations can be buffered by interacting with bacterial chaperones [108,109], yet another source of epistasis with unexplored consequences for AR.

There are very few studies investigating if epistasis occurs frequently during the evolution of multidrug resistant strains in clinical settings. Nevertheless, in clinical isolates of multidrug resistant *M. tuberculosis*, resistant to both rifampicin and ofloxacin, many carried a particular mutation known to confer ofloxacin resistance in the *gyrA* gene. This mutation has been shown in laboratory settings to have positive epistasis with several *rpoB* mutations (which confer rifampicin resistance) [99]. Clearly, further epidemiological studies are required to understand to which extent epistasis is relevant in clinical contexts.

Concluding remarks

Due to the high evolutionary potential of bacteria, the initial golden age of antibiotics to treat bacterial infections is quickly turning to a bronze age. *In vitro* and *in vivo* experimental evolution studies are fundamental to anticipate the evolutionary paths likely to be taken by potential pathogens upon exposure to drugs and to educate the society to the reality of microbial rapid evolutionary change. Currently, most studies of epistasis on AR rely on

observations between two resistance alleles or in between an AR mutation and the genetic background where it appears. The unfortunate reality of high frequency of multiple-resistance (e.g. clones carrying three and more resistances are becoming common), however, demands an understanding of higher order epistasis. This is a challenging task, but one that is urgently necessary. Profiting from the rapid evolution of bacteria in the lab, both to acquire multiple resistance and to compensate for resistance costs on fitness, experimental evolution studies focusing on key ecological and evolutionary factors (such as treatment duration, specific combinations of antibiotics and epistasis) may allow to more effectively manipulate and reduce the danger of multiple resistance.

It is also important to remember that fitness costs, compensation and epistatic effects are strongly environmental-dependent. Thus, further studies of competition, colonization, compensation and transmission using animal models are required (see **Outstanding Questions**). Such *in vivo* studies are likely essential to identify antibiotic targets that can hardly be compensated. Furthermore new surveys are required to quantify how pervasive epistasis is in clinical populations of pathogens. This knowledge would provide a theoretical framework for the development of novel antimicrobial strategies and therapeutic agents aiming at minimizing the evolution of multidrug resistance.

Acknowledgments

PD and RB were supported by Fundação para a Ciência e Tecnologia (FCT), fellowships SFRH/BPD/118474/2016 and SFRH/BPD/109517/2015, respectively. Current research is supported by project JPIAMR/0001/2016-ERA NET and ONEIDA project (LISBOA-01-0145-FEDER-016417) co-funded by FEEI - "Fundos Europeus Estruturais e de Investimento" from "Programa Operacional Regional Lisboa 2020" and by national funds from FCT - "Fundação para a Ciência e a Tecnologia.

BOX 1 – Antibiotic resistance conferred by mobile genetic elements

Mobile genetic elements carrying resistances (MRE) play a key role in the spread of AR, since they can disseminate in bacterial populations by horizontal gene transfer (HGT) [27]. MRE typically carry genes encoding functions that counteract the action of antibiotics by either enzymatic inactivation [110], efflux [111], synthesis of alternative enzymes to native targets [112] or target protection [113,114]. Three principal evolutionary mechanisms of HGT are

conjugation [115], transduction [116], and natural transformation [117], although alternative mechanisms have also been described [118].

Conjugation and transduction frequencies can be much higher *in vivo* than *in vitro* [29,119–122]. For example, in the context of the gut microbiota it was found that inflammation could greatly increase the rates of plasmid and bacteriophage transfer between *Salmonella* strains. Regarding natural transformation its prevalence is still not well quantified. Since the gut microbiota is a reservoir of AR genes [123,124], the HGT of MRE is likely frequent in the gut [125]. Among MRE, plasmids are probably the most clinically relevant [27]. The mechanisms underlying the effects of plasmid carriage on bacterial fitness in the absence of antibiotics remain poorly understood [52,126–128]. Interestingly, different plasmids cause diverse effects on bacterial fitness, ranging from large deleterious effects to no cost or even fitness advantage [52,126,127]. This heterogeneity can originate from plasmid features (size, resistance range, number of resistances, etc.), interference with the host physiology or interactions with the environment [129–131]. The fitness cost associated with plasmid carriage can be counterbalanced by acquiring compensatory mutations, either in the plasmid, in the bacterial chromosome, or in both [32,132–134]. These mutations often influence replication and transmission rates, impacting plasmid dissemination in bacterial populations [34,135]. Importantly, epistatic interactions between plasmids and chromosomal loci or other MRE [89,133,136–138] have been observed. Remarkably, these interactions include epistasis between plasmids and chromosomal resistance mutations [139], indicating that the acquisition of one resistance can favour or prevent the emergence of further resistance. Understanding the mechanisms underlying maintenance and dissemination of MRE in bacterial populations is thus essential to face the challenge of spreading ARs.

BOX 2 – Compensation through reversions

A particular case of compensation is reversion, when the adaptive mutation completely reverts the fitness costs by returning to the original genetic sequence. When reversion occurs in the presence of antibiotic, revertants are likely lost due to strong selection against them (alternatively, revertants can also be lost by genetic drift). Thus, reversion is considered to occur only in the absence of antibiotics and is clinically relevant since the bacteria re-gain sensitivity. However, compensation by acquiring additional mutations is far

more likely to occur than genetic reversion, since the range of targets for compensation is much broader [1].

Interestingly, phenotypic reversion (phenotypic sensitivity caused by the acquisition of an additional mutation, but maintaining the original resistance mutation) can also occur. For instance, mutations in the *rpsL* gene – encoding a ribosomal protein - confer resistance to streptomycin but several compensatory mutations occurring in other ribosomal proteins [140] or in translation elongation factors [141] can phenotypically revert resistance.

More recently, three studies have developed promising strategies to convert resistant bacteria into phenotypically sensitive to the original antibiotics [142–144]. The first study has re-sensitized resistant bacteria by treating it with a specifically designed oligonucleotide which acts as an antisense mRNA translation inhibitor and can be designed to target the mRNAs encoding resistance genes such as a constituent of the major drug efflux pump [142]. In the second study, a spiroisoxazoline family of Small Molecules Aborting Resistance (SMARt) was developed to phenotypically revert acquired resistance of *M. tuberculosis* to the prodrug ethionamide by inducing the expression of an alternative bioactivation pathway [143]. The SMARt molecule fully reversed ethionamide-acquired resistance and efficiently cleared an ethionamide-resistant infection in mice. In the third study, the assembly of functional membrane microdomains (structurally and functionally similar to lipid rafts of eukaryotic cells) of methicillin-resistant *S. aureus* (MRSA) was targeted and as a result resistance to penicillin was reverted both *in vitro* and *in vivo* [144].

Box 3 - Antibiotic resistance in light of Fisher's model of adaptation

Taking into account that the fitness effects of AR mutations are strongly dependent on the environment, it is of paramount importance to be able to anticipate the effect of resistance mutations across environments. Fisher's geometric model (FGM), which assumes a fitness landscape with a single peak, is a theoretical framework that allows predictions on the distribution of fitness effects (DFE) of mutations [145]. Under FGM an environmental change can be theoretically thought of as a change in the distance to the optimum of a given population or a change in the position of the optimum itself. FGM assumes that mutations affect pleiotropically a number of quantitative traits under stabilizing selection and many antibiotic targets are known to have pleiotropic effects. Maybe that is why this model has

been effective in describing the fitness effects of antibiotic resistance in the absence [49] and presence of antibiotic [17].

For instance, a study [49] using mutations in *E. coli* conferring resistance to streptomycin, rifampicin or D-cycloserine found that antibiotic mutation effects in the absence of antibiotic were well described by a shifted gamma distribution as predicted by FGM, with a shift parameter (reflecting the distance to the fitness peak) varying across environments. A somewhat extended FGM was also robust enough to accurately describe the mutational pattern of AR in *E. coli* across a gradient of nalidixic acid, a quinolone [17]. The implemented extensions took into account that: i) only a minor subset of mutations from specific regions of the genome will affect the ability to resist antibiotics (modularity). This proportion of resistance mutations seems to sharply decrease with the increase of the antibiotic concentration, a result with clinical relevance; ii) the effect of a mutation is dependent of the environmental selective constraints and thus, the same mutation may confer a fitness increase in one environment and not in others; and iii) different antibiotic concentrations may either constrain the optimal fitness that populations can reach (changing the height of the fitness peak) or change the rate of fitness increase with each mutation (changing the width of the peak). In the future, it will be important to distinguish in between these two latter processes.

Lastly, FGM also provides a reasonable theoretical framework to predict the dynamics of compensatory evolution of AR [146]. For instance, FGM predicts that compensatory mutations should occur at higher rates and cause higher fitness increases in strains where the costs of AR are larger.

Glossary

antibiotic resistance - an inheritable ability of microorganisms to grow at high concentrations of antibiotic (independently of whether it is bacteriostatic or bactericidal) and irrespective of the duration of treatment.

natural selection - evolutionary process by which the genotypes best phenotypically adapted to a particular environment in a population, increase in relative frequency with respect to less adapted organisms over generations.

fitness - a term that refers to the survival and reproductive success of an organism in an environment. In bacteria relative fitness is measured by competing two genotypes

(i.e.: resistant versus sensitive) and accounting for the change in frequency over time (competitive fitness). Fitness of bacteria can also be estimated by measuring reproductive related traits such as growth rate, carrying capacity or length of lag phase.

selective pressure – an evolutionary effect exerted by any cause or agent (i.e.: an antibiotic) that increases or reduces the reproductive success (fitness) of a genotype, changing its frequency in a population.

cost of resistance – deleterious effect to an organism fitness caused by the presence of either a chromosomal mutations conferring resistance or mobile genetic elements carrying resistance.

reversion – genetic reversion occurs when a mutation returns to the original genetic sequence. Phenotypic reversion of an AR mutation occurs when the resistance mutation is maintained but the sensitive phenotype is restored.

compensatory mutations – adaptive mutations which reduce the fitness costs caused by a pre-existing condition, such as the presence of antibiotic resistance mutations or MRE.

epistasis - phenomenon where the effect of one mutation is dependent on the presence of other pre-existing mutations, e.g. the genetic background.

FIGURE LEGENDS

Figure 1 – Emergence and maintenance of bacterial antibiotic resistance.

(A) Multidrug resistance under natural selection. *E. coli* can acquire rifampicin (Rif) and streptomycin (Str) resistance through mutations in the *rpoB* or *rpsL* genes, respectively (blue and purple circles), which allow the bacteria to survive during an antibiotic treatment (represented by the capsules). After antibiotic treatment, acquisition of resistance is often associated with fitness costs (red arrows) which can be alleviated (brown arrows) by compensatory mutations in known gene targets (orange circles). Bacterial population after rifampicin treatment will be enriched in resistance with compensated costs and, if submitted to subsequent treatments with other antibiotics (i.e.: streptomycin), may lead to the development of multiple resistances by the acquisition of mutations. **(B) Compensation under short-term and long-term antibiotic treatments**. Use of antibiotics can strongly select for resistant mutants **(a)**, favouring multiplication of the resistant strain **(b)**. On a long-term antibiotic treatment (*upper panel*), competition between resistant strains will increase over time and compensation to the fitness costs is likely to occur during the treatment **(c)**. In a short-term antibiotic treatment (*bottom panel*), compensation during treatment is unlikely because the advantage of resistance over the susceptible bacteria outweighs the fitness costs. In both scenarios, once the antibiotic treatment finishes, resistant strains will often have a fitness costs when competing against the susceptible strain and compensation will occur **(c)**. Time course of antibiotic treatment results in bacteria with different genetic backgrounds since they compensate differently for the costs of resistance. Whether this compensation occurred in presence or absence of antibiotics may strongly affect the fate of these mutants in competition with the sensitive strain.

Figure 2 – Genotype-by-genotype-by-environment (GxGxE) interactions.

(A) Epistasis between costly resistances. Epistasis can be *negative*, whereby the fitness of the double resistance is lower than expected, or *positive*, whereby the fitness of the double resistance is higher than expected. S*ign epistasis* represents a particular interaction, whereby the sign of the fitness of a double mutant changes depending on genetic background – a single mutation may be deleterious on the susceptible background, but may be beneficial or have no effect on a single resistance background. **(B) Epistasis between**

resistances changes compensation. When double resistance is not epistatic, the prediction is that the same compensation targets as the sum of the ones found in the single resistances will be found. When double resistance interacts negatively, increasing the fitness cost, a new set of compensatory mutations targeting the negative epistasis can occur [72]. When double resistance interacts positively, reducing the fitness cost, less compensatory mutations are expected to be available than the sum of targets found in the single resistances. Thickness of orange arrows represents compensatory mutations of higher effect and the numbers represent an example of expected compensatory genes for each resistance. **(C) Epistasis depends on the environment.** Fitness of double resistance (Ant_1^R + Ant_2^R) depends on the environment. Not only the same single resistances to either antibiotic (Ant_1^R, in green or Ant_2^R, in red) may have a different fitness depending on the environment but also the interactions in between Ant_1^R and Ant_2^R mutations might change depending on the environment, leading to negative epistasis in the environment I (*left panel*) and *positive epistasis* in environment II (*right panel*).

References

1 Andersson, D.I. and Hughes, D. (2010) Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat. Rev. Microbiol.* 8, 260–271

2 van Hoek, A.H.A.M. *et al.* (2011) Acquired Antibiotic Resistance Genes: An Overview. *Front. Microbiol.* 2, 10.3389/fmicb.2011.00203

3 Gullberg, E. *et al.* (2011) Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog.* 7, e1002158

4 MacLean, R.C. and Vogwill, T. (2015) Limits to compensatory adaptation and the persistence of antibiotic resistance in pathogenic bacteria. *Evol. Med. Public Health* 2015, 4–12

5 Bhullar, K. *et al.* (2012) Antibiotic resistance is prevalent in an isolated cave microbiome. *PloS One* 7, e34953

6 Forsberg, K.J. *et al.* (2012) The shared antibiotic resistome of soil bacteria and human pathogens. *Science* 337, 1107–1111

7 Charlesworth, B. (2010) *Elements of Evolutionary Genetics*, Roberts and Company Publishers.

8 Smith, R. and Coast, J. (2013) The true cost of antimicrobial resistance. *BMJ* 346, f1493

9 Hughes, D. and Andersson, D.I. (2017) Evolutionary Trajectories to Antibiotic Resistance. *Annu. Rev. Microbiol.* 71, 579–596

10 Wong, A. (2017) Epistasis and the Evolution of Antimicrobial Resistance. *Front. Microbiol.* 8, 246

11 Perfeito, L. *et al.* (2007) Adaptive mutations in bacteria: high rate and small effects. *Science* 317, 813–815

12 Martinez, J.L. and Baquero, F. (2000) Mutation Frequencies and Antibiotic Resistance. *Antimicrob. Agents Chemother.* 44, 1771–1777

13 Sousa, A. *et al.* (2017) Evolution of commensal bacteria in the intestinal tract of mice. *Curr. Opin. Microbiol.* 38, 114–121

14 Andersson, D.I. *et al.* (1998) Evidence that gene amplification underlies adaptive mutability of the bacterial lac operon. *Science* 282, 1133–1135

15 Roth, J.R. (2011) The joys and terrors of fast adaptation: new findings elucidate antibiotic resistance and natural selection. *Mol. Microbiol.* 79, 279–282

16 Cirz, R.T. *et al.* (2005) Inhibition of mutation and combating the evolution of antibiotic resistance. *PLoS Biol.* 3, e176

17 Harmand, N. *et al.* (2017) Fisher's geometrical model and the mutational patterns of antibiotic resistance across dose gradients. *Evol. Int. J. Org. Evol.* 71, 23–37

18 Rodríguez-Verdugo, A. *et al.* (2013) Evolution of Escherichia coli rifampicin resistance in an antibiotic-free environment during thermal stress. *BMC Evol. Biol.* 13, 50

19 Andersson, D.I. and Hughes, D. (2014) Microbiological effects of sublethal levels of antibiotics. *Nat. Rev. Microbiol.* 12, 465–478

20 Miller, J.H. *et al.* (1999) Direct selection for mutators in Escherichia coli. *J. Bacteriol.* 181, 1576–1584

21 Vulić, M. *et al.* (1997) Molecular keys to speciation: DNA polymorphism and the control of genetic exchange in enterobacteria. *Proc. Natl. Acad. Sci. U. S. A.* 94, 9763–9767

22 Denamur, E. *et al.* (2005) Intermediate Mutation Frequencies Favor Evolution of Multidrug Resistance in Escherichia coli. *Genetics* 171, 825–827

23 Andersson, D.I. (2015) Improving predictions of the risk of resistance development against new and old antibiotics. *Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.* 21, 894–898

24 Woodford, N. and Ellington, M.J. (2007) The emergence of antibiotic resistance by mutation. *Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.* 13, 5–18

25 Lynch, M. *et al.* (2016) Genetic drift, selection and the evolution of the mutation rate. *Nat. Rev. Genet.* 17, 704

26 Rolo, J. *et al.* (2017) Evidence for the evolutionary steps leading to mecA-mediated β-lactam resistance in staphylococci. *PLOS Genet.* 13, e1006674

27 Andersson, D.I. and Hughes, D. (2017) Selection and Transmission of Antibiotic-Resistant Bacteria. *Microbiol. Spectr.* 5, MTBP-0013-2016
28 Hernando-Amado, S. *et al.* (2017) Fitness costs associated with the acquisition of antibiotic resistance. *Essays Biochem.* 61, 37–48
29 Dionisio, F. *et al.* (2002) Plasmids spread very fast in heterogeneous bacterial communities. *Genetics* 162, 1525–1532
30 Lee, S.W. and Edlin, G. (1985) Expression of tetracycline resistance in pBR322 derivatives reduces the reproductive fitness of plasmid-containing Escherichia coli. *Gene* 39, 173–180
31 McDermott, P.J. *et al.* (1993) Adaptation of Escherichia coli growth rates to the presence of pBR322. *Lett. Appl. Microbiol.* 17, 139–143
32 Dahlberg, C. and Chao, L. (2003) Amelioration of the cost of conjugative plasmid carriage in Eschericha coli K12. *Genetics* 165, 1641–1649
33 Enne, V.I. *et al.* (2004) Enhancement of host fitness by the sul2-coding plasmid p9123 in the absence of selective pressure. *J. Antimicrob. Chemother.* 53, 958–963
34 Heuer, H. *et al.* (2007) Frequent conjugative transfer accelerates adaptation of a broad-host-range plasmid to an unfavorable Pseudomonas putida host. *FEMS Microbiol. Ecol.* 59, 738–748
35 Marciano, D.C. *et al.* (2007) A fitness cost associated with the antibiotic resistance enzyme SME-1 beta-lactamase. *Genetics* 176, 2381–2392
36 Starikova, I. *et al.* (2013) Fitness costs of various mobile genetic elements in Enterococcus faecium and Enterococcus faecalis. *J. Antimicrob. Chemother.* 68, 2755–2765
37 Hall, A.R. *et al.* (2015) Costs of antibiotic resistance - separating trait effects and selective effects. *Evol. Appl.* 8, 261–272
38 Seppälä, H. *et al.* (1997) The Effect of Changes in the Consumption of Macrolide Antibiotics on Erythromycin Resistance in Group A Streptococci in Finland. *N. Engl. J. Med.* 337, 441–446
39 Enne, V.I. *et al.* (2001) Persistence of sulphonamide resistance in Escherichia coli in the UK despite national prescribing restriction. *Lancet Lond. Engl.* 357, 1325–1328
40 Bean, D.C. *et al.* (2005) Resistance among Escherichia coli to sulphonamides and other antimicrobials now little used in man. *J. Antimicrob. Chemother.* 56, 962–964
41 Gottesman, B.S. *et al.* (2009) Impact of quinolone restriction on resistance patterns of Escherichia coli isolated from urine by culture in a community setting. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* 49, 869–875
42 Miskinyte, M. and Gordo, I. (2013) Increased survival of antibiotic-resistant Escherichia coli inside macrophages. *Antimicrob. Agents Chemother.* 57, 189–195
43 Durão, P. *et al.* (2016) Enhanced Survival of Rifampin- and Streptomycin-Resistant Escherichia coli Inside Macrophages. *Antimicrob. Agents Chemother.* 60, 4324–4332
44 Hershberg, R. (2017) Antibiotic-Independent Adaptive Effects of Antibiotic Resistance Mutations. *Trends Genet. TIG* 33, 521–528
45 Skurnik, D. *et al.* (2013) Enhanced in vivo fitness of carbapenem-resistant oprD mutants of Pseudomonas aeruginosa revealed through high-throughput sequencing. *Proc. Natl. Acad. Sci. U. S. A.* 110, 20747–20752
46 Roux, D. *et al.* (2015) Fitness cost of antibiotic susceptibility during bacterial infection. *Sci. Transl. Med.* 7, 297ra114
47 Koch, G. *et al.* (2014) Evolution of resistance to a last-resort antibiotic in Staphylococcus aureus via bacterial competition. *Cell* 158, 1060–1071
48 Durão, P. *et al.* (2015) Multiple Resistance at No Cost: Rifampicin and Streptomycin a Dangerous Liaison in the Spread of Antibiotic Resistance. *Mol. Biol. Evol.* 32, 2675–2680
49 Trindade, S. *et al.* (2012) Antibiotic resistance and stress in the light of Fisher's model. *Evol. Int. J. Org. Evol.* 66, 3815–3824
50 Brandis, G. *et al.* (2015) Comprehensive phenotypic characterization of rifampicin resistance mutations in Salmonella provides insight into the evolution of resistance in Mycobacterium tuberculosis. *J. Antimicrob. Chemother.* 70, 680–685

51 O'Neill, A.J. *et al.* (2006) Molecular genetic and structural modeling studies of Staphylococcus aureus RNA polymerase and the fitness of rifampin resistance genotypes in relation to clinical prevalence. *Antimicrob. Agents Chemother.* 50, 298–309

52 Vogwill, T. and MacLean, R.C. (2015) The genetic basis of the fitness costs of antimicrobial resistance: a meta-analysis approach. *Evol. Appl.* 8, 284–295

53 Baym, M. *et al.* (2016) Spatiotemporal microbial evolution on antibiotic landscapes. *Science* 353, 1147–1151

54 Chevereau, G. *et al.* (2015) Quantifying the Determinants of Evolutionary Dynamics Leading to Drug Resistance. *PLOS Biol.* 13, e1002299

55 Liu, A. *et al.* (2011) Selective advantage of resistant strains at trace levels of antibiotics: a simple and ultrasensitive color test for detection of antibiotics and genotoxic agents. *Antimicrob. Agents Chemother.* 55, 1204–1210

56 Vos, M.G.J. de *et al.* (2017) Interaction networks, ecological stability, and collective antibiotic tolerance in polymicrobial infections. *Proc. Natl. Acad. Sci.* 114, 10666–10671

57 Radlinski, L. *et al.* (2017) Pseudomonas aeruginosa exoproducts determine antibiotic efficacy against Staphylococcus aureus. *PLOS Biol.* 15, e2003981

58 Palmer, A.C. *et al.* (2010) Chemical decay of an antibiotic inverts selection for resistance. *Nat. Chem. Biol.* 6, 105–107

59 Gomez, J.E. *et al.* (2017) Ribosomal mutations promote the evolution of antibiotic resistance in a multidrug environment. *eLife* 6, e20420

60 Meredith, H.R. *et al.* (2015) Collective antibiotic tolerance: mechanisms, dynamics and intervention. *Nat. Chem. Biol.* 11, 182–188

61 Zhang, H. *et al.* (2013) Genome sequencing of 161 Mycobacterium tuberculosis isolates from China identifies genes and intergenic regions associated with drug resistance. *Nat. Genet.* 45, 1255–1260

62 Comas, I. *et al.* (2011) Whole-genome sequencing of rifampicin-resistant Mycobacterium tuberculosis strains identifies compensatory mutations in RNA polymerase genes. *Nat. Genet.* 44, 106–110

63 Maisnier-Patin, S. *et al.* (2002) Compensatory adaptation to the deleterious effect of antibiotic resistance in Salmonella typhimurium. *Mol. Microbiol.* 46, 355–366

64 Reynolds, M.G. (2000) Compensatory evolution in rifampin-resistant Escherichia coli. *Genetics* 156, 1471–1481

65 Qi, Q. *et al.* (2016) The genomic basis of adaptation to the fitness cost of rifampicin resistance in Pseudomonas aeruginosa. *Proc. Biol. Sci.* 283, 20152452

66 Besier, S. *et al.* (2005) Compensatory adaptation to the loss of biological fitness associated with acquisition of fusidic acid resistance in Staphylococcus aureus. *Antimicrob. Agents Chemother.* 49, 1426–1431

67 Björkman, J. *et al.* (2000) Effects of Environment on Compensatory Mutations to Ameliorate Costs of Antibiotic Resistance. *Science* 287, 1479–1482

68 Allen, R.C. *et al.* (2017) Reversing resistance: different routes and common themes across pathogens. *Proc. Biol. Sci.* 284,

69 Maisnier-Patin, S. and Andersson, D.I. (2004) Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. *Res. Microbiol.* 155, 360–369

70 Levin, B.R. *et al.* (2000) Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. *Genetics* 154, 985–997

71 Moura de Sousa, J. *et al.* (2015) Potential for adaptation overrides cost of resistance. *Future Microbiol.* 10, 1415–1431

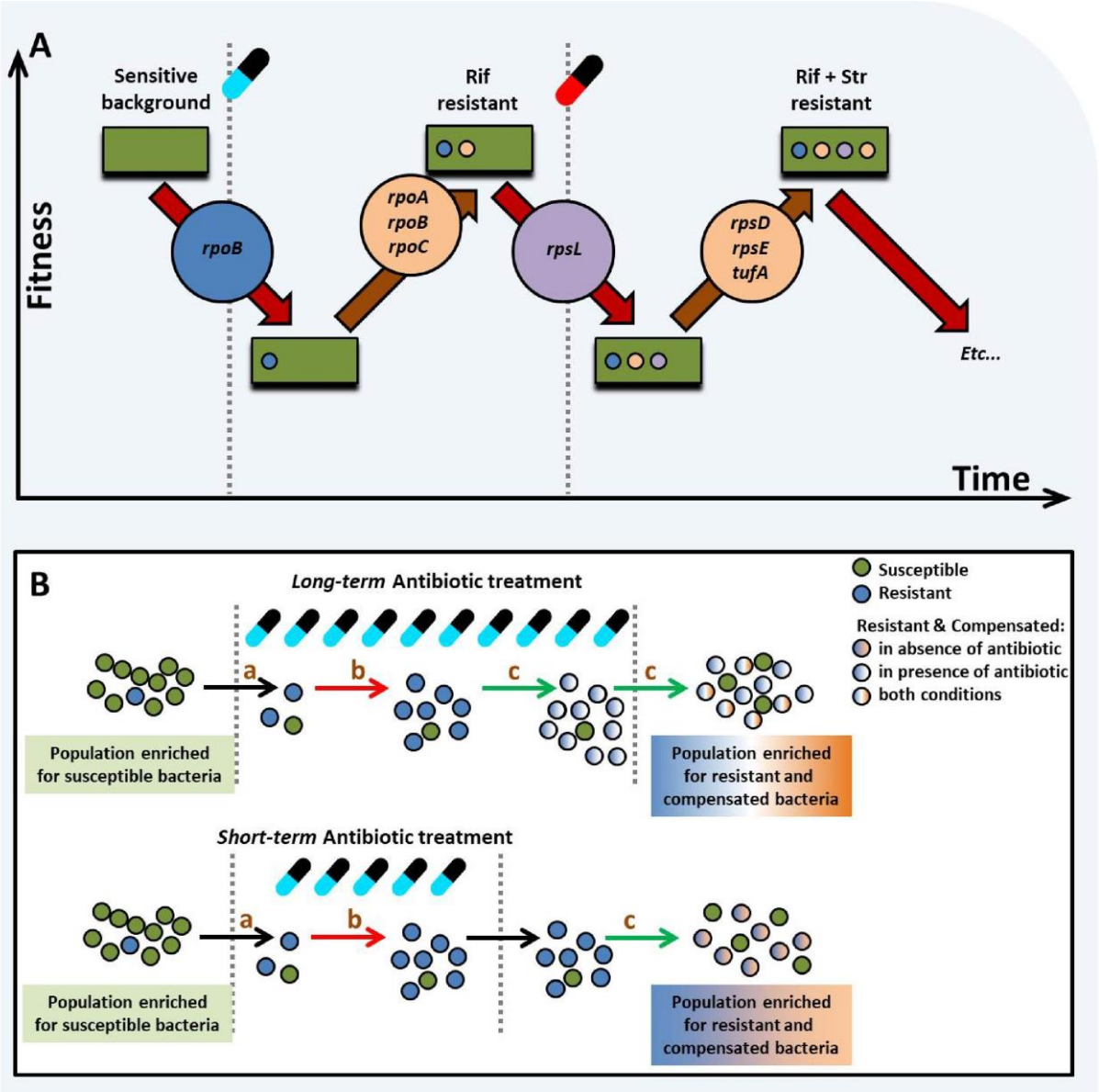
72 Moura de Sousa, J. *et al.* (2017) Multidrug-resistant bacteria compensate for the epistasis between resistances. *PLoS Biol.* 15, e2001741

73 Couce, A. and Tenaillon, O.A. (2015) The rule of declining adaptability in microbial evolution experiments. *Front. Genet.* 6, 10.3389/fgene.2015.00099

74 Marcusson, L.L. *et al.* (2009) Interplay in the selection of fluoroquinolone resistance and bacterial fitness. *PLoS Pathog.* 5, e1000541
75 Rozen, D.E. *et al.* (2007) Fitness costs of fluoroquinolone resistance in Streptococcus pneumoniae. *Antimicrob. Agents Chemother.* 51, 412–416
76 Paulander, W. *et al.* (2007) Multiple mechanisms to ameliorate the fitness burden of mupirocin resistance in Salmonella typhimurium. *Mol. Microbiol.* 64, 1038–1048
77 Llewelyn, M.J. *et al.* (2017) The antibiotic course has had its day. *BMJ* 358, j3418
78 Lambert, H.P. (1999) Don't keep taking the tablets? *Lancet Lond. Engl.* 354, 943–945
79 Gagneux, S. *et al.* (2006) The competitive cost of antibiotic resistance in Mycobacterium tuberculosis. *Science* 312, 1944–1946
80 Chang, H.-H. *et al.* (2015) Origin and Proliferation of Multiple-Drug Resistance in Bacterial Pathogens. *Microbiol. Mol. Biol. Rev. MMBR* 79, 101–116
81 Coscolla, M. *et al.* (2015) Genomic epidemiology of multidrug-resistant Mycobacterium tuberculosis during transcontinental spread. *J. Infect. Dis.* 212, 302–310
82 Björkholm, B. *et al.* (2001) Mutation frequency and biological cost of antibiotic resistance in Helicobacter pylori. *Proc. Natl. Acad. Sci. U. S. A.* 98, 14607–14612
83 Luo, N. *et al.* (2005) Enhanced in vivo fitness of fluoroquinolone-resistant Campylobacter jejuni in the absence of antibiotic selection pressure. *Proc. Natl. Acad. Sci. U. S. A.* 102, 541–546
84 MacLean, R.C. *et al.* (2010) Diminishing returns from beneficial mutations and pervasive epistasis shape the fitness landscape for rifampicin resistance in Pseudomonas aeruginosa. *Genetics* 186, 1345–1354
85 Humphrey, B. *et al.* (2012) Fitness of Escherichia coli strains carrying expressed and partially silent IncN and IncP1 plasmids. *BMC Microbiol.* 12, 53
86 Di Luca, M.C. *et al.* (2017) Low biological cost of carbapenemase-encoding plasmids following transfer from Klebsiella pneumoniae to Escherichia coli. *J. Antimicrob. Chemother.* 72, 85–89
87 Trindade, S. *et al.* (2009) Positive Epistasis Drives the Acquisition of Multidrug Resistance. *PLoS Genet.* 5, e1000578
88 Weinreich, D.M. *et al.* (2006) Darwinian evolution can follow only very few mutational paths to fitter proteins. *Science* 312, 111–114
89 San Millan, A. *et al.* (2014) Positive epistasis between co-infecting plasmids promotes plasmid survival in bacterial populations. *ISME J.* 8, 601–612
90 Weinreich, D.M. *et al.* (2005) Perspective: Sign epistasis and genetic constraint on evolutionary trajectories. *Evol. Int. J. Org. Evol.* 59, 1165–1174
91 Schenk, M.F. *et al.* (2013) Patterns of Epistasis between beneficial mutations in an antibiotic resistance gene. *Mol. Biol. Evol.* 30, 1779–1787
92 Salverda, M.L.M. *et al.* (2011) Initial mutations direct alternative pathways of protein evolution. *PLoS Genet.* 7, e1001321
93 Woods, R.J. *et al.* (2011) Second-order selection for evolvability in a large Escherichia coli population. *Science* 331, 1433–1436
94 Blount, Z.D. *et al.* (2012) Genomic analysis of a key innovation in an experimental Escherichia coli population. *Nature* 489, 513–518
95 Szendro, I.G. *et al.* (2013) Predictability of evolution depends nonmonotonically on population size. *Proc. Natl. Acad. Sci. U. S. A.* 110, 571–576
96 Müller, B. *et al.* (2013) The heterogeneous evolution of multidrug-resistant Mycobacterium tuberculosis. *Trends Genet. TIG* 29, 160–169
97 Ward, H. *et al.* (2009) The cost of multiple drug resistance in Pseudomonas aeruginosa. *J. Evol. Biol.* 22, 997–1003
98 Angst, D.C. and Hall, A.R. (2013) The cost of antibiotic resistance depends on evolutionary history in Escherichia coli. *BMC Evol. Biol.* 13, 163
99 Borrell, S. *et al.* (2013) Epistasis between antibiotic resistance mutations drives the evolution of extensively drug-resistant tuberculosis. *Evol. Med. Public Health* 2013, 65–74

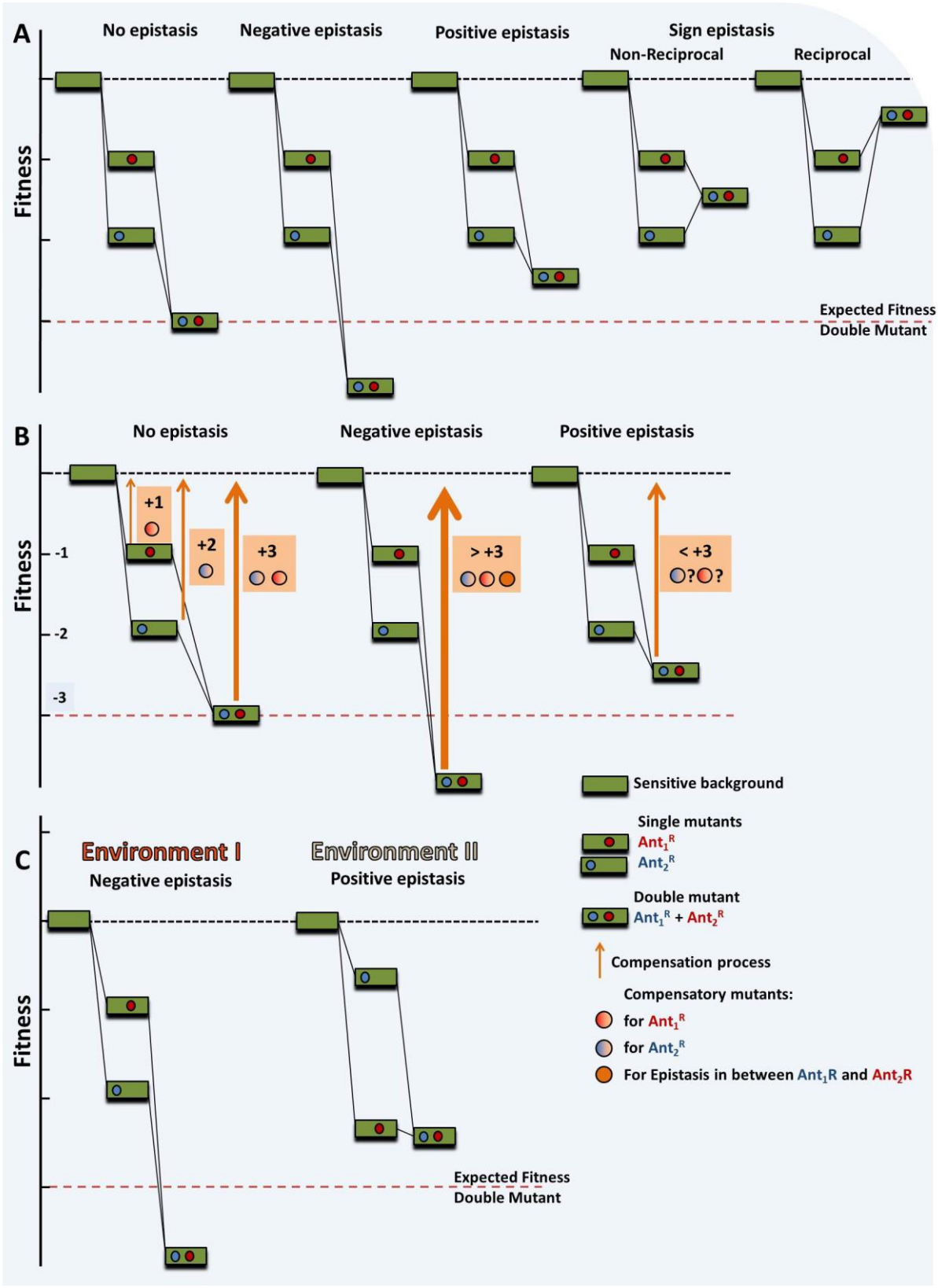
100 Palmer, A.C. and Kishony, R. (2013) Understanding, predicting and manipulating the genotypic evolution of antibiotic resistance. *Nat. Rev. Genet.* 14, 243–248
101 Bloom, J.D. *et al.* (2005) Thermodynamic prediction of protein neutrality. *Proc. Natl. Acad. Sci. U. S. A.* 102, 606–611
102 Jacquier, H. *et al.* (2013) Capturing the mutational landscape of the beta-lactamase TEM-1. *Proc. Natl. Acad. Sci. U. S. A.* 110, 13067–13072
103 Gros, P.-A. *et al.* (2009) The evolution of epistasis and its links with genetic robustness, complexity and drift in a phenotypic model of adaptation. *Genetics* 182, 277–293
104 Blanquart, F. *et al.* (2014) Properties of selected mutations and genotypic landscapes under Fisher's geometric model. *Evol. Int. J. Org. Evol.* 68, 3537–3554
105 Hwang, S. *et al.* (2017) Genotypic Complexity of Fisher's Geometric Model. *Genetics* 206, 1049–1079
106 Wang, X. *et al.* (2002) Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs. *J. Mol. Biol.* 320, 85–95
107 Zhang, W. *et al.* (2012) Multidimensional epistasis and fitness landscapes in enzyme evolution. *Biochem. J.* 445, 39–46
108 Maisnier-Patin, S. *et al.* (2005) Genomic buffering mitigates the effects of deleterious mutations in bacteria. *Nat. Genet.* 37, 1376–1379
109 Tokuriki, N. and Tawfik, D.S. (2009) Chaperonin overexpression promotes genetic variation and enzyme evolution. *Nature* 459, 668–673
110 Wright, G.D. (2005) Bacterial resistance to antibiotics: enzymatic degradation and modification. *Adv. Drug Deliv. Rev.* 57, 1451–1470
111 Poole, K. (2005) Efflux-mediated antimicrobial resistance. *J. Antimicrob. Chemother.* 56, 20–51
112 Wellington, E.M.H. *et al.* (2013) The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria. *Lancet Infect. Dis.* 13, 155–165
113 Nguyen, F. *et al.* (2014) Tetracycline antibiotics and resistance mechanisms. *Biol. Chem.* 395, 559–575
114 Redgrave, L.S. *et al.* (2014) Fluoroquinolone resistance: mechanisms, impact on bacteria, and role in evolutionary success. *Trends Microbiol.* 22, 438–445
115 Llosa, M. *et al.* (2002) Bacterial conjugation: a two-step mechanism for DNA transport. *Mol. Microbiol.* 45, 1–8
116 Touchon, M. *et al.* (2017) Embracing the enemy: the diversification of microbial gene repertoires by phage-mediated horizontal gene transfer. *Curr. Opin. Microbiol.* 38, 66–73
117 Blokesch, M. (2017) In and out—contribution of natural transformation to the shuffling of large genomic regions. *Curr. Opin. Microbiol.* 38, 22–29
118 García-Aljaro, C. *et al.* (2017) Beyond the canonical strategies of horizontal gene transfer in prokaryotes. *Curr. Opin. Microbiol.* 38, 95–105
119 Maisonneuve, null *et al.* (2000) Effects of yoghurt intake on plasmid transfer and colonisation with transconjugants in the digestive tract of mice associated with human faecal flora. *FEMS Microbiol. Ecol.* 31, 241–248
120 Stecher, B. *et al.* (2012) Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae. *Proc. Natl. Acad. Sci. U. S. A.* 109, 1269–1274
121 De Paepe, M. *et al.* (2016) Carriage of λ Latent Virus Is Costly for Its Bacterial Host due to Frequent Reactivation in Monoxenic Mouse Intestine. *PLoS Genet.* 12, e1005861
122 Diard, M. *et al.* (2017) Inflammation boosts bacteriophage transfer between Salmonella spp. *Science* 355, 1211–1215
123 Hu, Y. *et al.* (2013) Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. *Nat. Commun.* 4, 2151
124 van Schaik, W. (2015) The human gut resistome. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 370, 20140087

125 Huddleston, J.R. (2014) Horizontal gene transfer in the human gastrointestinal tract: potential spread of antibiotic resistance genes. *Infect. Drug Resist.* 7, 167–176
126 Mc Ginty, S.É. and Rankin, D.J. (2012) The evolution of conflict resolution between plasmids and their bacterial hosts. *Evol. Int. J. Org. Evol.* 66, 1662–1670
127 Baltrus, D.A. (2013) Exploring the costs of horizontal gene transfer. *Trends Ecol. Evol.* 28, 489–495
128 San Millan, A. and MacLean, R.C. (2017) Fitness Costs of Plasmids: a Limit to Plasmid Transmission. *Microbiol. Spectr.* 5, 10.1128/microbiolspec.MTBP-0016-2017
129 Ender, M. *et al.* (2004) Fitness cost of SCCmec and methicillin resistance levels in Staphylococcus aureus. *Antimicrob. Agents Chemother.* 48, 2295–2297
130 Jalasvuori, M. *et al.* (2011) Bacteriophage selection against a plasmid-encoded sex apparatus leads to the loss of antibiotic-resistance plasmids. *Biol. Lett.* 7, 902–905
131 Kempf, A.J. *et al.* (2016) Multiple Plasmids Contribute to Antibiotic Resistance and Macrophage Survival In Vitro in CMY2-Bearing Salmonella enterica. *Foodborne Pathog. Dis.* 13, 398–404
132 Morton, E.R. *et al.* (2013) Large deletions in the pAtC58 megaplasmid of Agrobacterium tumefaciens can confer reduced carriage cost and increased expression of virulence genes. *Genome Biol. Evol.* 5, 1353–1364
133 San Millan, A. *et al.* (2014) Positive selection and compensatory adaptation interact to stabilize non-transmissible plasmids. *Nat. Commun.* 5, 5208
134 Yano, H. *et al.* (2016) Evolved plasmid-host interactions reduce plasmid interference cost. *Mol. Microbiol.* 101, 743–756
135 De Gelder, L. *et al.* (2008) Adaptive plasmid evolution results in host-range expansion of a broad-host-range plasmid. *Genetics* 178, 2179–2190
136 Morton, E.R. *et al.* (2014) Non-additive costs and interactions alter the competitive dynamics of co-occurring ecologically distinct plasmids. *Proc. Biol. Sci.* 281, 20132173
137 Gama, J.A. *et al.* (2017) Conjugation efficiency depends on intra and intercellular interactions between distinct plasmids: Plasmids promote the immigration of other plasmids but repress co-colonizing plasmids. *Plasmid* 93, 6–16
138 Werisch, M. *et al.* (2017) Conjugative plasmids enable the maintenance of low cost non-transmissible plasmids. *Plasmid* 91, 96–104
139 Silva, R.F. *et al.* (2011) Pervasive sign epistasis between conjugative plasmids and drug-resistance chromosomal mutations. *PLoS Genet.* 7, e1002181
140 Böck, A. *et al.* (1979) Ribosomal ambiguity (ram) mutations facilitate dihydrostreptomycin binding to ribosomes. *FEBS Lett.* 104, 317–321
141 Kraal, B. *et al.* (1995) Antibiotic resistance mechanisms of mutant EF-Tu species in Escherichia coli. *Biochem. Cell Biol. Biochim. Biol. Cell.* 73, 1167–1177
142 Ayhan, D.H. *et al.* (2016) Sequence-Specific Targeting of Bacterial Resistance Genes Increases Antibiotic Efficacy. *PLoS Biol.* 14, e1002552
143 Blondiaux, N. *et al.* (2017) Reversion of antibiotic resistance in Mycobacterium tuberculosis by spiroisoxazoline SMARt-420. *Science* 355, 1206–1211
144 García-Fernández, E. *et al.* (2017) Membrane Microdomain Disassembly Inhibits MRSA Antibiotic Resistance. *Cell* 171, 1354–1367.e20
145 Martin, G. and Lenormand, T. (2015) The fitness effect of mutations across environments: Fisher's geometrical model with multiple optima. *Evol. Int. J. Org. Evol.* 69, 1433–1447
146 Sousa, A. *et al.* (2012) Cost of Antibiotic Resistance and the Geometry of Adaptation. *Mol. Biol. Evol.* 29, 1417–1428


A
Sensitive background
Rif resistant
Rif + Str resistant
rpoB
rpoA rpoB rpoC
rpsL
rpsD rpsE tufA
Etc...
Fitness
Time
B
Long-term Antibiotic treatment
Susceptible
Resistant
Resistant & Compensated:
in absence of antibiotic
in presence of antibiotic
both conditions
a
b
c
c
Population enriched for susceptible bacteria
Population enriched for resistant and compensated bacteria
Short-term Antibiotic treatment
a
b
c
Population enriched for susceptible bacteria
Population enriched for resistant and compensated bacteria

842

843


A
No epistasis
Negative epistasis
Positive epistasis
Sign epistasis
Non-Reciprocal
Reciprocal
Fitness
Expected Fitness
Double Mutant
B
No epistasis
Negative epistasis
Positive epistasis
Fitness
-1
-2
-3
+1
+2
+3
> +3
< +3
?
?
C
Environment I
Negative epistasis
Environment II
Positive epistasis
Fitness
Expected Fitness
Double Mutant
Sensitive background
Single mutants
Ant1R
Ant2R
Double mutant
Ant1R + Ant2R
Compensation process
Compensatory mutants:
for Ant1R
for Ant2R
For Epistasis in between Ant1R and Ant2R

844

845

Highlights

- Most antibiotic resistance mutations reduce bacterial fitness in the absence of the antibiotic, but some are not costly, or can even be advantageous in certain environments, including infection-related conditions.
- Acquiring a new resistance can alleviate the cost of a pre-existing one, thus favouring the emergence of multidrug resistant bacteria.
- The compensatory evolution of multidrug resistant bacteria is distinct from that of single-resistant bacteria, since the proteins mediating functional interactions between those affected by resistance mutations become new targets for their compensation.

Outstanding Questions

- Fitness effects of AR are environmental dependent. How to identify the key characteristics of the environment to be able to predict resistance effects *in vivo*?
- Compensation of costs of multiple resistances can occur in a few days in the lab. What is the rate at which compensation occurs in the human host?
- How many mutations are adaptive to pathogens depending on the presence or absence of antibiotics in the environment?
- To what extent is epistasis relevant *in vivo* and how to measure epistasis between many resistances?