Evolutionary mechanisms shaping the maintenance of antibiotic resistance

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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 \( \sim 10^5 \) 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 \( 10^{-8} \)
$9 \times 10^{-10}$ 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 \textit{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].

\textbf{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 \textit{E. coli} and \textit{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 \textit{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. 1B 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.
1B 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 2A 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 2A). 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 reversion 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 2B). 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.

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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 \(M.\, tuberculosi\)s 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 \textit{in vitro} and \textit{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. Sign 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$^R_1 + $ Ant$^R_2$) depends on the environment. Not only the same single resistances to either antibiotic (Ant$^R_1$, in green or Ant$^R_2$, in red) may have a different fitness depending on the environment but also the interactions in between Ant$^R_1$ and Ant$^R_2$ 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).
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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?