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Review article

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Translating eco-evolutionary biology into therapy to tackle antibiotic resistance

Fernando Sanz-García[1], Teresa Gil-Gil[2,3], Pablo Laborda[2,4,5], Paula Blanco[6,7], Luz-Edith Ochoa-Sánchez[2], Fernando Baquero[8], José Luis Martínez[2] ✉ & Sara Hernando-Amado[2] ✉

Abstract

Antibiotic resistance is currently one of the most important public health problems. The golden age of antibiotic discovery ended decades ago, and new approaches are urgently needed. Therefore, preserving the efficacy of the antibiotics currently in use and developing compounds and strategies that specifically target antibiotic-resistant pathogens is critical. The identification of robust trends of antibiotic resistance evolution and of its associated trade-offs, such as collateral sensitivity or fitness costs, is invaluable for the design of rational evolution-based, ecology-based treatment approaches. In this Review, we discuss these evolutionary trade-offs and how such knowledge can aid in informing combination or alternating antibiotic therapies against bacterial infections. In addition, we discuss how targeting bacterial metabolism can enhance drug activity and impair antibiotic resistance evolution. Finally, we explore how an improved understanding of the original physiological function of antibiotic resistance determinants, which have evolved to reach clinical resistance after a process of historical contingency, may help to tackle antibiotic resistance.

Sections

[1]Departamento de Microbiología, Medicina Preventiva y Salud Pública, Universidad de Zaragoza, Zaragoza, Spain. [2]Centro Nacional de Biotecnología, CSIC, Darwin 3, Madrid, Spain. [3]Programa de Doctorado en Biociencias Moleculares, Universidad Autónoma de Madrid, Madrid, Spain. [4]The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kongens Lyngby, Denmark. [5]Department of Clinical Microbiology, 9301, Rigshospitalet, Copenhagen, Denmark. [6]Molecular Basis of Adaptation, Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain. [7]VISAVET Health Surveillance Centre, Universidad Complutense Madrid, Madrid, Spain. [8]Department of Microbiology, Hospital Universitario Ramón y Cajal (IRYCIS), CIBER en Epidemiología y Salud Pública (CIBERESP), Madrid, Spain.
✉e-mail: jlmtnez@cnb.csic.es; shernando@cnb.csic.es

Introduction

The problem of antibiotic resistance has been mainly addressed through two approaches: the development of new antibiotics and the restriction of the use of those currently available. Few antibiotics are presently under development, and a reduction in the use of antibiotics, mainly in high-income countries, has not been enough to curb the rise of antibiotic resistance. Indeed, antibiotic resistance is the consequence of natural selection and genetic drift, primarily exerted on bacterial populations. In this respect, antibiotic resistance is the unavoidable outcome of the historical contingency and the adaptive determinism of bacterial genes and genomes in the presence of selective antibiotics[1,2]. This means that, although restriction policies are certainly needed, they can only slow the emergence and spread of antibiotic resistance[3]. For this reason, knowing the evolutionary pathways most likely to be selected in the presence of an antibiotic and the eco-evolutionary consequences of antibiotic resistance for bacterial physiology (Box 1) can aid in diminishing the emergence and spread of antibiotic resistance, thus having a positive impact on human health. In fact, it has been proposed that the exploitation of functional trade-offs associated with the acquisition of resistance, such as fitness costs and collateral sensitivity[4–9], might optimize the utilization of antibiotics currently in use for treating infections. Further, understanding the effects of antibiotic resistance on bacterial physiology, most specifically on bacterial metabolism, may aid in designing metabolic interventions favouring the efficacy of antibiotics, particularly their efficacy against antibiotic-resistant bacteria[10,11]. In addition, the analysis of the functional networks to which antibiotic resistance determinants belong and the understanding of their original functions, not limited to conferring antibiotic resistance, constitute a scarcely explored area that may help to identify anti-virulence drugs as well as compounds that might be used as adjuvants for improving the activity of antibiotics[12].

Genetic drift, a process that leads to the non-deterministic emergence or disappearance of a variant genotype, is complementary to the process of natural selection. Experimental evolution studies have shown that resistance can evolve even in the absence of antibiotics[13], although certainly these resistant organisms can be efficiently enriched by antibiotic exposure. In natural selection the time axis prevails, and a simple, relatively predictable 'cause–effect' process from the ancestor to the evolved cell can theoretically be achieved. By contrast, complexity is the hallmark of genetic drift, in which the space axis provides a huge number of opportunities for the bacterial cells to be transmitted and to occupy different, and frequently changing, microenvironments in which any stochastic variant can be selected[14]. In addition, bacteria have access to new genetic information provided by mobile genetic elements from the local microbial community.

In this conceptual landscape (Fig. 1), the simplest object of natural selection or genetic drift is the individual bacterial cell, each one being extremely complex per se. Hence, any selected genetic variation necessarily influences bacterial physiology and, therefore, may affect the basic bacterial replication rate (fitness). This final outcome influences bacterial microecological interactions. However, complexity is not necessarily linked to randomness and, therefore, to unpredictability. Random variations might display preferential (robust) paths in which randomness is rather hampered by the complexity of multidimensional influences (for example, metabolism, transcriptional networks or epigenetics)[1]. In this Review, we explore the possibility of identifying and predicting some critical evolutionary trade-offs with practical applications for antibiotic therapy or for prevention of the emergence of bacterial antibiotic-resistant variants (Box 1). We also

Box 1

Methods to analyse the mutational evolution of antibiotic resistance and its consequences

Mutationally acquired antibiotic resistance has been traditionally analysed by direct selection of resistant mutants and by serial passage adaptive laboratory evolution (ALE) assays[153]. The first method enables fast detection of single-step mutations present in bacterial populations at any stage, by plating 10^9–10^{10} bacterial cells on the selective drug. The mutations can be identified via whole-genome sequencing, and their importance for human health is evaluated by inspecting repositories containing sequenced genomes of resistant clinical isolates of the bacterium under study[154]. The capacity of direct selection to detect resistance can be boosted by resorting to high-throughput techniques that permit site-directed mutagenesis of specific DNA segments[155–158].

The direct-selection methodologies enable analysis of the effect of mutations at each site of the genome on antibiotic resistance, but they do not predict the ones that are more likely to be fixed in a bacterial population when growing in the presence of an antibiotic — that is, the most probable evolutionary trajectories towards antibiotic resistance. To reach that goal, serial passage ALE experiments are a more accurate approach (Fig. 3). In classic ALE assays, replicate populations of a parental strain, sometimes a hypermutator to accelerate the selection of potential mutations[159], are subcultured daily in fresh media over a long time span, with increases in the antibiotic concentration every few days. These experiments are useful to predict possible combinations of resistance mutations that can be selected by a drug, along with their order of acquisition and potential clinical relevance[31,160,161]. However, they are not the most appropriate method to look for robust evolutionary trade-offs associated with the acquisition of antibiotic resistance that might be exploited to tackle resistance in clinical settings. It has been reported that shorter ALE assays, without increases of drug concentration, using replicate populations of different resistant mutants of the same bacterial species[39,40,58] or even distinct clinical isolates[41], are a much more appropriate tool to study robust evolutionary trade-offs, such as collateral sensitivity, with potential in vivo application (Fig. 3). Strategies defined by this approach may aid in designing sequential or combined treatments against heterogeneous bacterial infections.

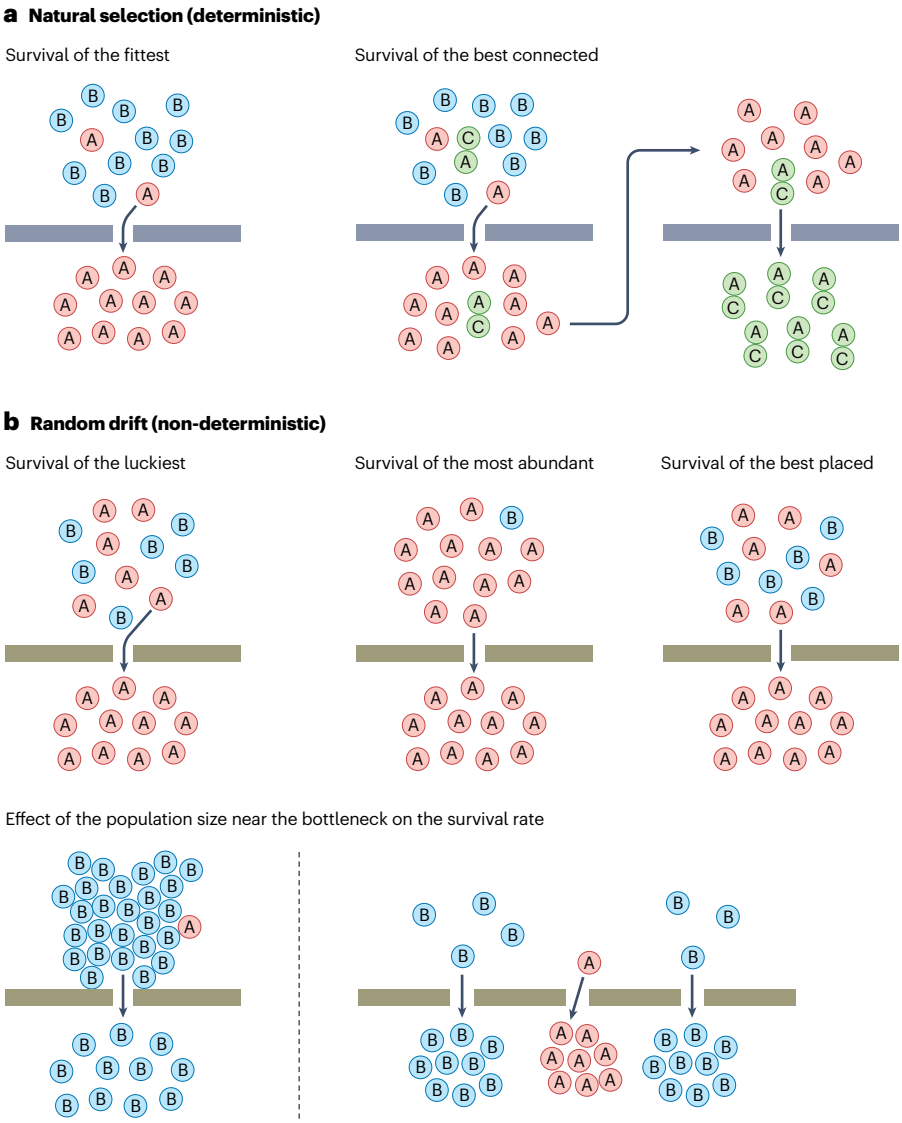


Fig. 1 | Natural selection and random drift in the evolution of antibiotic resistance. a, Altered population sizes following a bottleneck (indicated by a hole between two rectangles) due to natural selection. The change in frequency of organisms harbouring two different alleles (A and B) when crossing bottlenecks is shown. Only the population having an adaptive genotype (A), such as antibiotic resistance, is able to give rise to a progeny after exposure to the selective event (survival of the fittest) (left panel). A non-adaptive genotype (C) is selected because of its connection with the selected genotype (survival of the best connected) (middle panel). **b**, Genetic drift. A and B are present in similar proportions, and just by chance, A crosses the bottleneck before B and gives rise to a progeny (survival of the luckiest) (top-left panel). A is much more abundant than B in the original population, thus it is more likely to survive across the bottleneck (survival of the most abundant) (top-middle panel). Just by chance, A is at near the hole in the bottleneck at a given time and it crosses the bottleneck first (survival of the best placed) (top-right panel). The effect of the population size near the bottleneck on the survival rate is also shown: if B is very abundant, A has almost no chance of surviving beyond the bottleneck (bottom-left panel), but if the population size is low, A might compete with B and give rise to a progeny (bottom-right panel).

discuss how targeting bacterial metabolism can impair antibiotic resistance evolution and increase the activity of antibiotics. Further, we argue that a deeper knowledge of the original role of antibiotic resistance determinants may aid in enhancing antibiotic activity and preventing resistance emergence. It is worth mentioning that, besides being a problem for human health, antibiotic resistance is one of the few evolutionary processes that can be experimentally addressed and that is amenable to being studied over a short period of time. We also discuss how the study of antibiotic resistance evolution affects the general postulates of evolutionary biology (Box 2).

Identifying robust evolutionary trade-offs

As mentioned above, antibiotic effectiveness is currently compromised by bacterial evolution. Therefore, evolution-based approaches that exploit the trade-offs associated with antibiotic resistance acquisition may help to tackle this problem, mainly by helping to achieve a more effective use of currently available antibiotics[15]. Among those exploitable evolutionary trade-offs, collateral sensitivity and fitness costs are the most relevant (Fig. 2).

Collateral sensitivity is a phenomenon by which the acquisition of resistance to one drug leads to an increased susceptibility to a second one[16]. Although recent work[7] shows that this trade-off may emerge when antibiotic resistance is acquired by horizontal gene transfer, the most comprehensive studies of collateral sensitivity have been related to the acquisition of mutation-driven antibiotic resistance[5,8,16,17]. Hence, in this Review, we mainly focus on trade-offs associated with the mutational acquisition of antibiotic resistance.

The identification of collateral sensitivity patterns may help to define drug pairs that, used either in combination or sequentially alternated, are more efficient than when they are used individually[18] (Fig. 3). In addition, it may also improve the activity of other antibiotics and diminish the de novo evolution of resistance[19]. In this regard, different studies have addressed this issue, finding collateral sensitivity patterns associated with the use of specific antibiotics and suggesting

Box 2

Studying the evolution of antibiotic resistance to understand general laws of evolution

Evolution theories have been traditionally defined based on inferences derived from the observation of the fossil record, but their validation is not something that is regularly experimentally addressed. Microorganisms constitute the perfect model for such evolutionary studies given their large population sizes and their fast replication rate. Indeed, the debate between the theories of Lamarck and Darwin regarding whether mutations precede adaptation or whether phenotypic adaptation is transferred to the offspring was solved, using a bacterial model, by Luria and Delbrück, who showed that mutations conferring resistance to bacteriophages were present in bacterial populations before being infected by these viruses[162]. Further, the use of a minimal infection model has enabled the experimental validation of the Haldane hypothesis, which proposes that resistance to infections is the selecting force for some prevalent inherited diseases[163].

The experimental study of antibiotic resistance therefore has a heuristic value because it helps to explain evolution in general terms and to experimentally validate evolutionary theories. The contributions of critical elements in evolution, such as co-selection, epistasis, mutation rates, population bottlenecks, relative fitness and selective pressure, have been explored in bacterial studies about antibiotic resistance, and authoritative reviews on this topic have been published[164]. In addition, more recent work on collateral sensitivity robustness is helping to reveal processes of parallel evolution[39], convergent evolution[40] and, quite notably, the stability of phenotypes (such as collateral sensitivity) that are co-selected but are not adaptive to the applied selective force. These processes may explain some evolutionary emergencies that appear without a clear, specific selective force behind them. In fact, they could be just the consequence of the stability of trade-offs associated with the selection of the actual adaptive phenotype[58]. Also within this area, there has been a strong debate on the emergence of complex structures such as feathers, which are only useful when the structure is complete and hence for which stepwise evolution does not seem reasonable. To solve this paradox, it has been proposed that exaptation, a process by which the acquisition of a new function is the consequence of a switch of habitat and not of a genetic change, might underline these evolutionary trajectories[165]. Notably, it has been shown that intrinsic antibiotic resistance genes have functions that go beyond antibiotic resistance[96]. However, when they are transferred to new organisms, they lose the biochemical, physiological and ecological context in which they evolved and the only function they have is to confer antibiotic resistance. This gain-of-function of mobile antibiotic resistance genes provides experimental evidence that exaptation is a relevant process in evolution.

a clinical potential in combination or sequential therapies[5,6,20–26]. However, in most cases, the collateral sensitivity patterns detected are not conserved among different isolates or even among replicated populations of the same clone[27–29], and recent work has shown that they are not always stable[30]. The reason for this lack of robustness is that epistatic and pleiotropic phenomena shape the evolution of antibiotic resistance and restrict the mutations (and their associated trade-offs) that can be selected in a particular genetic background[31,32]. Indeed, long-term evolution experiments have shown that antibiotic resistance evolution is contingent on the genomic background[33]. Differences in the genomic background do not need to be large; in fact, it has been shown in *Pseudomonas aeruginosa* that loss-of-function mutations in a single gene (*lasR*), which encodes a regulator of the quorum-sensing response, modify the collateral sensitivity patterns of said pathogen[31]. Noteworthy, *lasR* inactivation not infrequently occurs in bacteria infecting patients with cystic fibrosis[34]. A recent publication has shown that, even when mutations in the same gene provide antibiotic resistance, collateral sensitivity might be allele specific, making the emergence and prediction of robust collateral sensitivity patterns even more difficult[35].

This lack of conservation of collateral sensitivity hinders the clinical exploitation of this trade-off, unless robust — in other words, predictable — collateral sensitivity patterns displayed by different isolates are identified. For this purpose, the exploration of available information might reveal antibiotic pairs with disjoint resistance, understood as a situation in which resistance to one antibiotic is commonly associated with susceptibility to another and vice versa, at the population level. Nevertheless, the analysis of a large dataset comprising 448,563 antimicrobial susceptibility tests showed that disjoint resistance is rarely conserved at the species level[36]. Further, in the few cases in which disjoint resistance showed some conservation, this conservation was not due to the existence of robust collateral sensitivity[37]. This means that the identification of robust collateral sensitivity patterns (Box 1) requires specific experimental analysis (Fig. 3). The first aspect to be explored is the conservation of collateral sensitivity in pre-existing antibiotic-resistant mutants, a feature poorly explored until recently. Indeed, infections, particularly chronic infections, may involve heterogeneous bacterial populations, including different antibiotic-resistant mutants that have been selected during previous treatments[38]. For a combined and/or sequential collateral sensitivity-based treatment to be faithfully useful, it should be effective against the different resistant mutants that might be present in the site of infection. Despite this situation, most work in the field has been limited to the study of a single, antibiotic-susceptible, model strain. Recent work is filling this reductionist knowledge gap. For instance, the analysis of a set of different resistant mutants of *P. aeruginosa* PA14 enabled the identification of robust collateral sensitivity patterns associated with the use of either ceftazidime or ciprofloxacin[39,40]. In the case of ceftazidime resistance, this phenotype was the result of parallel evolution due to the selection of the same genetic event, and selection was independent of the nature of the pre-existing mutant[39]. In the case of ciprofloxacin resistance, collateral sensitivity was associated with the

acquisition of different ciprofloxacin resistance mutations: a case of phenotypic convergence in the absence of parallel evolution[40]. Based on this evolutionary information, the alternation of ceftazidime with tobramycin, and the combination of ciprofloxacin with tobramycin or aztreonam, have been proposed to treat *P. aeruginosa* infections[39,40]. It is worth stating that, although ciprofloxacin resistance rendered a robust collateral sensitivity to aztreonam and tobramycin, the opposite did not happen: neither aztreonam nor tobramycin mutational resistance rendered a robust collateral sensitivity to any of the antibiotics tested. It has been proposed that cycling strategies could be designed based on information on reciprocal collateral sensitivity[6]. However, the results stated above imply that collateral sensitivity patterns are not always bijective and that the order of antibiotics used during a treatment matters.

In the mentioned studies, the effect of pre-existing mutations in the conservation of collateral sensitivity was explored. However, these were isogenic mutants, and to implement these collateral sensitivity-based therapeutic approaches in the clinic, the robustness of the phenotype should emerge at the species level and not just at the strain level, as species are the routine diagnostic targets. In fact, it has been shown that, occasionally, the conservation of collateral sensitivity might depend more on the mutations involved than on the genomic background[17]. This has been recently explored by using clinical isolates of *P. aeruginosa* with different genomic backgrounds and different ab initio mutational resistomes, including representatives of high-risk epidemic clones, that underwent short-term evolution in the presence of ciprofloxacin[41]. Interestingly, although different ciprofloxacin resistance mutations were acquired (depending on the genomic background and the original pre-existing mutational resistome), the use of this drug led to a robust collateral sensitivity to tobramycin and aztreonam. In line with these findings, it has been described that ciprofloxacin-resistant clinical isolates of *P. aeruginosa* present a convergent increased susceptibility to aminoglycosides[23]. This supports the notion that this evolutionary trade-off could be clinically exploited to successfully treat *P. aeruginosa* infections by using the combinations of ciprofloxacin–tobramycin or ciprofloxacin–aztreonam[40,41].

One issue that emerges from these studies is that phenotypic convergence towards collateral sensitivity can be conserved even if the acquisition of resistance is due to different mutations, in this case, conferring resistance to ciprofloxacin[40,41]. Therefore, another question that arises is whether different mutations selected by different antibiotic families might also render a common collateral sensitivity pattern. In fact, this particular situation has been observed in *P. aeruginosa*-resistant mutants selected in the presence of ceftazidime, tobramycin or tigecycline. All of these mutants presented collateral sensitivity to fosfomycin, owing to parallel changes in expression of the gene encoding the intrinsic fosfomycin-inactivating enzyme FosA and of the genes encoding enzymes from the alternative peptidoglycan recycling pathway[42]. This indicates that robust collateral sensitivity patterns may emerge even when different selective forces are applied, a feature that has also been described in the case of mutants resistant to different antibiotics, all of them presenting nitrofurantoin collateral sensitivity[43]. This indicates that it would be possible to alternate or combine different drugs with a second fixed antibiotic. The identification of these broad-spectrum collateral sensitivity patterns could be useful for implementing more efficient therapeutic approaches against bacterial infections.

We have discussed the need to find robust collateral sensitivity patterns in bacteria having different genetic backgrounds. However, this desired robustness also implies that the same collateral sensitivity phenotype is faithfully expected to emerge when bacteria are challenged with a specific antibiotic in environments with a different nutritional composition, as may occur in diverse body locations. Recent studies have shown that the nutritional composition may shape, to a certain degree, the evolution of both antibiotic resistance and collateral sensitivity[44,45]. The reason is that each antibiotic resistance mutation may lead to a different resistance level and/or a different fitness depending on the composition of the medium (that is, urine, synthetic sputum or rich laboratory medium). Therefore, antibiotic resistance mutational range and collateral sensitivity are habitat-dependent; growing in urine during urinary tract infections or in sputum, in the case of lung infections, renders different evolutionary antibiotic resistance patterns[44,45]. Consequently, the identification of robust collateral sensitivity patterns that emerge in different genetic backgrounds and are preserved regardless of the nutritional composition of the habitat will be an important advance in tackling antibiotic resistance. A final aspect to be taken into consideration is the possible conservation of collateral sensitivity, not just among members of the same species but also among different species. This feature has begun to be explored[46,47]. Although the results are still scarce, these studies can be an important step forward for the implementation of global collateral sensitivity-based therapeutic approaches.

Collateral sensitivity has been traditionally studied as a trade-off associated with the selection of mutants conferring antibiotic resistance to a first drug. However, transient antibiotic resistance can emerge when bacteria grow in the presence of inducers of the production of antibiotic resistance determinants. Recent work has shown that it is possible to intentionally induce this situation, causing different genetic backgrounds of *P. aeruginosa* (including clinical strains) to present a robust collateral sensitivity pattern[48]. This work provides evidence that collateral sensitivity can be achieved and exploited without the need to select antibiotic-resistant mutants.

A second trade-off of antibiotic resistance evolution that could be exploited for tackling this urgent health issue is fitness cost (Fig. 2). It has been generally accepted that antibiotic resistance acquisition

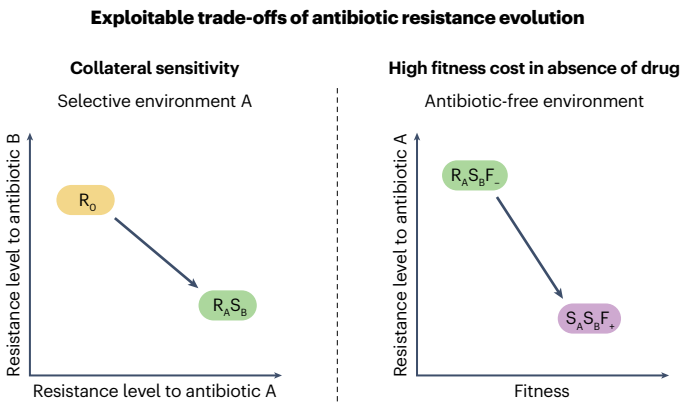
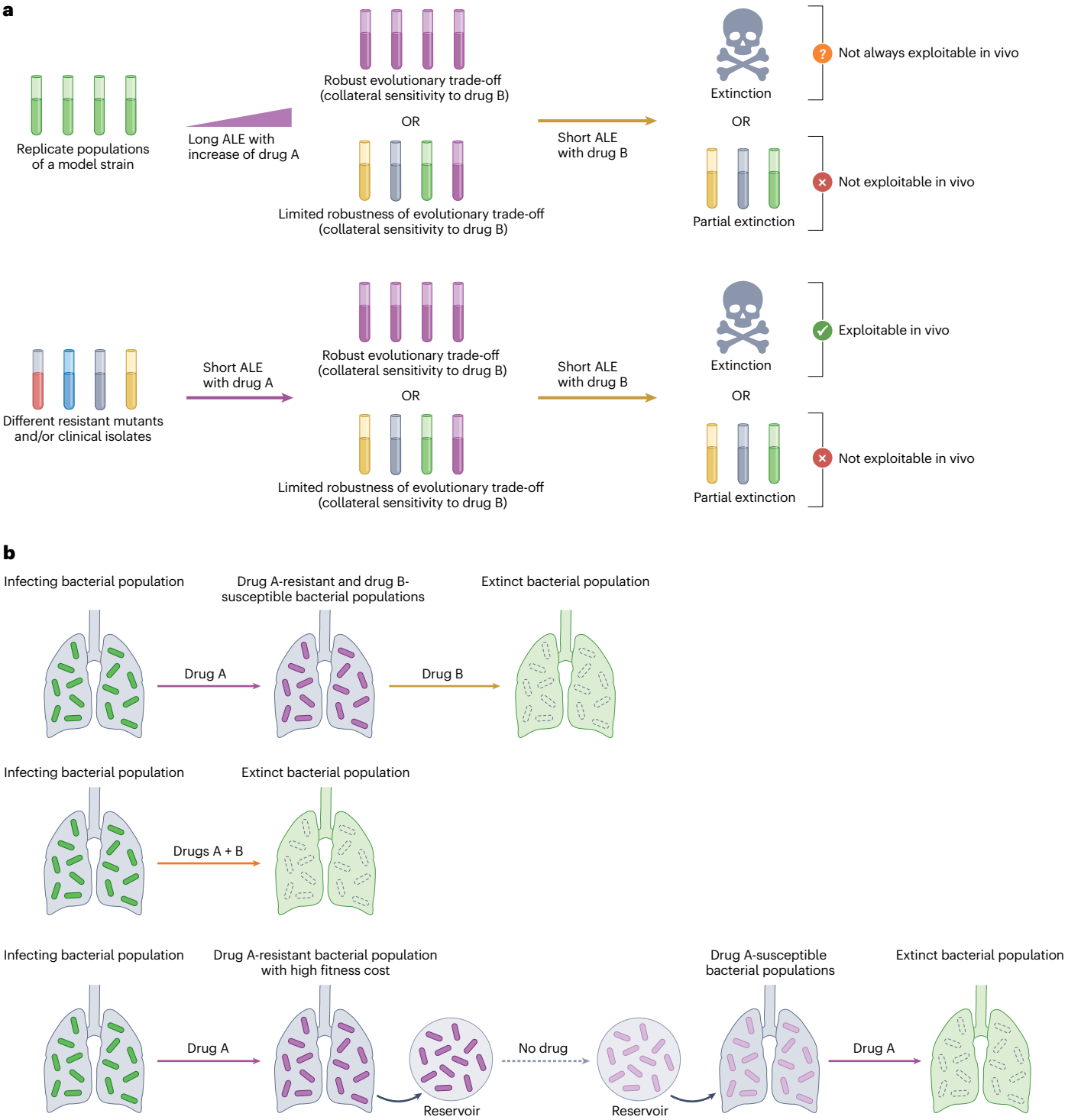


Fig. 2 | Exploitable trade-offs of antibiotic resistance evolution. Exposure to an antibiotic A may select resistance (R) to this drug and collateral sensitivity (S) to an antibiotic B (selective environment A). Reversion of resistance to A and preservation of collateral sensitivity to B, both acquired in A environments, may occur in antibiotic-free environments when the fitness (F) cost of resistance to A is compensated for (antibiotic-free environment). F_+, high fitness; F_-, low fitness; R_0, resistance before selection.



is associated with an impaired fitness of the resistant bacteria when growing in antibiotic-free environments. This led to the proposal that the alternation of antibiotics with periods of drug restriction could be a promising approach, as resistant strains would be replaced by susceptible ones in the absence of antibiotics. Unfortunately, different trials implementing this approach have shown little success[49]. The main reason is that not all antibiotic resistance mutations entail a substantial fitness cost and that, when it does happen, compensation may occur through selection of compensatory mutations, reversion of the original mutations or non-mutational compensation through metabolic rewiring[50–53]. In fact, low-cost resistance could be selected in the long term as part of the evolutionary process, turning resistant bacteria into commensal but non-susceptible organisms inhabiting many environments. In addition, the drugs to be restricted are mainly chosen on the basis of

Fig. 3 | Methodologies to detect robust evolutionary trade-offs associated with antibiotic resistance and therapeutic strategies based on these robust patterns. a, Classic serial passage adaptive laboratory evolution (ALE) experiments with replicate populations of a model strain and increasing drug concentrations are not always an efficient way to look for robust evolutionary trade-offs associated with antibiotic resistance that could be exploited in vivo (top rows). By contrast, short ALE assays without increases in drug concentrations, and using diverse resistant mutants or clinical strains, emulate more accurately the heterogeneous infections that can be found in vivo, hence possibly enabling the design of combination or sequential therapies against them if robust evolutionary trade-offs are found (bottom rows) (see Box 1 for more details). The consequences of two sequential ALE assays are shown. In the first, the populations are challenged with one antibiotic (A) and resistant mutants, presenting collateral sensitivity to another antibiotic (B), are selected (represented by purple). In the second, populations are challenged with B. As shown, extinction is only achieved when collateral sensitivity is robust. **b**, Bacterial populations (homogeneous and heterogeneous) can be driven to extinction by alternation or combination of drugs A and B if they have collateral sensitivity to drug B associated with the use of drug A (top and middle rows). If resistance to drug A is robustly unstable in the absence of selective pressure, the alternation of drug restriction periods with the use of drug A can be a feasible approach to deal with bacterial infections (bottom row).

weak cause–effect associations; for instance, it should be highlighted that old drugs might select for resistance to newer antibiotics[54]. It is important to note that, until now, antibiotic cycling policies have been empirically designed[55]. They have not been based — although they should be — on experimental information on the fitness costs associated with resistance to a given drug, nor on the possible compensation (or lack of compensation) of these fitness costs[55].

Despite this situation, cycling strategies still remain as a potential approach to curb antibiotic resistance. Until now, the trials of this approach have been not regularly based on previous evolutionary-driven information. However, the finding of robust patterns of antibiotic resistance instability may help in the implementation of evidence-based, evolution-driven cycling strategies[55] (Fig. 3). In this regard, it is worth mentioning that antibiotic resistance decline in the absence of selection is drug specific[56,57] and may depend on both the initial fitness cost and the original genetic background in which those fitness costs are compensated for[58], as epistatic interactions restrict both antibiotic resistance evolution[32,59–61] and compensatory evolution. Therefore, the identification of drugs for which antibiotic resistance may be unstable when selection ceases is of huge relevance to successful application of drug restriction periods. For example, it has been described that the compensatory evolution of fitness costs of ceftazidime-resistant mutants with different genetic backgrounds leads to a rapid decline of resistance in antibiotic-free environments[58]. Notably, although antibiotic resistance declined, an antibiotic resistance-associated robust pattern of collateral sensitivity (collateral sensitivity to tobramycin) remained. Nevertheless, it is important to notice that fitness restoration of bacterial antibiotic resistance is not always associated with the preservation of collateral sensitivity networks, a feature recently described for clinical isolates of ciprofloxacin-resistant *Escherichia coli*[30]. The identification of robust patterns of antibiotic resistance decline in the absence of selection, ideally linked to the preservation of the existent collateral sensitivity phenotypes, may help in the design of therapeutic strategies based on the alternation of an antibiotic with periods of drug restriction. For that, and based on the scant experimental data available on this topic[57,58], we propose that detailed information is needed on the possible mutations, or the most frequent mutations, that could be selected in the presence of a specific drug and on the possible decline of resistance and collateral sensitivity stability that could occur in the absence of selective pressure.

Targeting bacterial metabolism

Recent work has revealed that mutations in metabolic genes can be the underlying cause of evolution towards antibiotic resistance[62–64], which supports the idea that developing antibiotic resistance drives metabolic adaptations. In turn, metabolism also influences evolutionary trajectories to acquire antibiotic resistance, as has been demonstrated for *E. coli*. Antibiotic resistance develops faster when *E. coli* grows on glucose than when growing on acetate. This suggests a greater metabolic plasticity during respiratory–fermentative metabolism than during obligatory respiratory metabolism on acetate, which facilitates the acquisition of antibiotic resistance in this microorganism[65]. Further, *E. coli* presents an increasing mutation rate and distinct mutational range when growing anaerobically[66]. Furthermore, a depressed tricarboxylic acid (TCA) cycle contributes to the acquisition of ampicillin resistance in *Edwardsiella piscicida*[67]. We cannot disregard the fact that in some of these circumstances, the emergence of mutations can be favoured in stress-induced persister cells with phenotypically reduced susceptibility or in heteroresistant populations[68,69].

Differences have been reported in the patterns of resistance acquisition, and in fitness cost compensation, as a function of growing conditions, including those that bacteria encounter during infections. The reasons behind these findings are the differential fitness costs and levels of resistance associated with each mutation in each local ecosystem[44,70]. Hence, to evaluate the potential exploitation of robust trade-offs associated with the acquisition of resistance, antibiotic resistance evolution must be explored under different growth conditions. Among them, the most relevant ones are those that bacteria face during infection, because pathoadaptive changes, some of them linking virulence with metabolism and antibiotic resistance, can occur in this situation. This is the case with the facultative intracellular pathogen *Listeria monocytogenes*, a bacterium that is classified as intrinsically resistant to fosfomycin under laboratory conditions. A key element for *L. monocytogenes* virulence is Hpt, a glucose-6-phosphate transporter that enables the intracellular growth of this bacterial pathogen and whose expression is induced under such conditions. Notably, this transporter is also the port of entry of fosfomycin; thus, its induction renders bacteria susceptible to fosfomycin during intracellular infection[71].

It is clear that the pathogen–antibiotic in vivo interaction occurs in a complex environment including phagocytes, molecules of innate and adaptive immunity and, eventually, a growth-limiting chemosphere of antimicrobial metabolites of host or bacterial origin[72]. Moreover, this ecosystem frequently presents critical limitations in organic nutrient and iron availability, together with particular physicochemical conditions such as specific pH or oxidation–reduction potential values. All of these elements may have an impact on antibiotic resistance. For instance, it has been described that dormant *Mycobacterium tuberculosis* cells are susceptible to metronidazole, an antibiotic used to treat anaerobic bacterial infections, when oxygen is gradually depleted — a condition that can be encountered during latent tuberculosis[73]. Interestingly, this drug prevents the reactivation of latent *M. tuberculosis* infection in macaque models[74], despite *M. tuberculosis* being considered an aerobic bacterium.

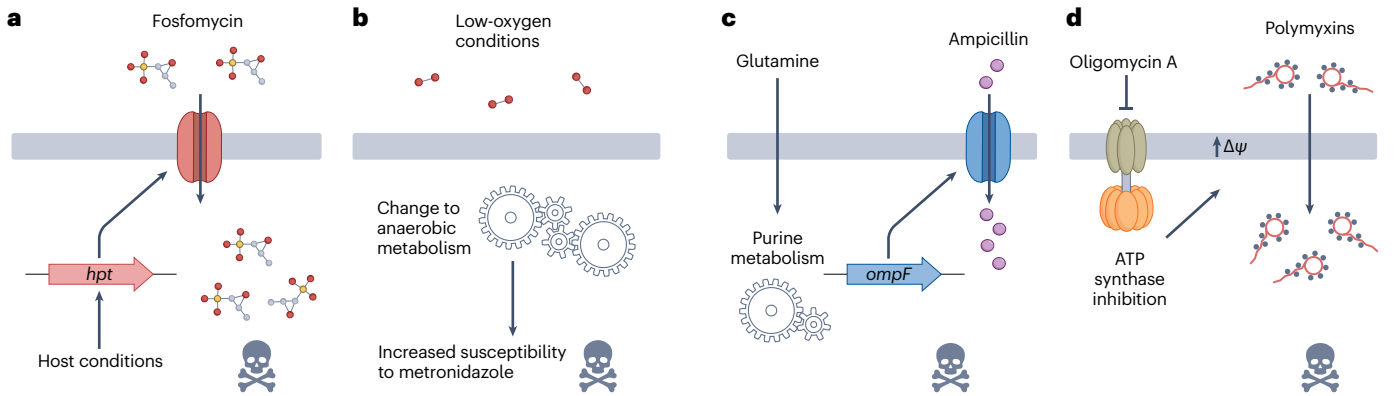


Fig. 4 | Examples of bacterial metabolic changes that impair antimicrobial resistance. a,**b**, Infection conditions can trigger metabolic changes that render bacteria susceptible to certain antibiotics. That is the case for *Listeria monocytogenes*, which is more susceptible to fosfomycin when growing intracellularly owing to the induction of the expression of the Hpt transporter, which is needed for intracellular growth but also enables fosfomycin to enter the cell (part **a**). Low oxygen levels present during latent tuberculosis conditions can make *Mycobacterium tuberculosis* more susceptible to the drug metronidazole (part **b**). **c**,**d**, Metabolic interference can be exploited to improve the activity of certain antibiotics or repurposing the use of others. This has been observed in multidrug-resistant *Escherichia coli* strains, in which addition of glutamine induces ampicillin uptake through the overproduction of the OmpF transporter stimulated by the purine metabolic pathway (part **c**). Inhibition of the *Staphylococcus aureus* ATP synthase by adding oligomycin A leads to membrane hyperpolarization that can facilitate the activity of polymyxins (part **d**). $\Delta\psi$, membrane potential.

Having in mind that antibiotic resistance evolution depends on the bacterial metabolic state, it can be proposed that interfering with bacterial metabolism could be exploited to improve the activity of available antibiotics and to reduce the emergence of antibiotic resistance[75]. Some examples of the situations or metabolic interventions that affect antibiotic resistance are shown in Fig. 4. To define these elements, transcriptomic, proteomic, metabolomic and metabolic modelling studies comparing susceptible and resistant strains are instrumental[76]. This is the case of *Staphylococcus aureus* vancomycin-intermediate strains that develop vancomycin resistance due to metabolic adaptations, such as an incremented acetogenesis or a decreased TCA cycle activity. Combination therapies based on the use of vancomycin with amino sugars or purines have been tested to kill these mutants[77]. Similarly, it has been described that the metabolism of alanine, aspartate and glutamate was inactivated, and glutamine metabolism was repressed, in multidrug-resistant (MDR) *E. coli*. Notably, the addition of glutamine stimulates ampicillin influx to reach intracellular concentrations high enough to kill MDR strains of *E. coli*, *P. aeruginosa*, *Acinetobacter baumannii* or *Klebsiella pneumoniae* in a mouse infection model[78]. More recently, it has been shown that different antibiotic-resistant *E. coli* mutants from a single strain, selected in the presence of different antibiotics, display convergent changes in their transcriptomes, possibly through convergent regulatory rewiring of the multidrug transport system. Notably, these changes are associated with an increased susceptibility to various antimicrobial peptides[79]. Whether these findings can be generalized to clinical isolates and/or to other bacterial species remains to be established.

It has also been shown that persistent bacterial cells might become susceptible to antibiotics by increasing their metabolic activity following the administration of NAD^+-boosting compounds or metabolites that increase TCA cycle activity[10]. In addition, it has also been shown that glutamate increases aminoglycoside efficacy by priming the activity of the pyruvate cycle, which provides respiratory energy, in *E. coli*[80]. Interestingly, understanding metabolic changes associated with antibiotic resistance may help in developing strategies to impair antibiotic resistance evolution. It has been shown that tetracycline-resistant *E. coli* incur fitness costs and undergo strong changes in their physiology, including downregulation of the TCA cycle and disruption of redox homeostasis, to support use of the proton motive force for tetracycline efflux. These fitness costs are compensated for by the activity of the global regulator ArcA. Notably, the inhibition of ArcA by sertraline, a drug that generates a similar metabolome profile as an *arcA*-knockout strain, is synergistic with tetracycline, strongly reducing the fitness of tetracycline-resistant cells[81].

Metabolic interference might also be useful for the repurposing of antibiotics, particularly for the treatment of bacteria that are considered resistant to them. For instance, Gram-positive bacteria, such as *S. aureus*, are considered intrinsically resistant to polymyxins. However, the inhibition of ATP synthase, which is the main source of energy during respiration, sensitizes *S. aureus* to polymyxins. This suggests that the alteration of *S. aureus* metabolism by inhibiting this enzyme would enable the use of this group of antibiotics against *S. aureus* infections[82]. Further, it has been recently shown that the pharmacological modification of thiamine metabolism improves the activity of several antibiotics against *P. aeruginosa*[83]. Furthermore, a decreased membrane potential caused by a reduction in the activity of the sodium-translocating NADH–ubiquinone oxidoreductase (Na^+–NQR) complex confers aminoglycoside resistance. This change in antibiotic susceptibility is due to a reduced amount of alanine. Nevertheless, this phenotype can be reverted by the addition of exogenous L-alanine, which promotes aminoglycoside-mediated killing[84]. Moreover, glucose, or its combination with glycine, serine or threonine, has been shown to revert kanamycin resistance in *E. piscicida* through the activation of the TCA cycle and the promotion of amino acid biosynthesis[85,86].

Although most studies on the effect of metabolic alterations on antibiotic resistance evolution have focused on nutritional aspects,

recent work has shown that metabolic alterations may also produce structural changes that might also alter antibiotic resistance evolution. Methicillin resistance in *S. aureus* is subjected to regulation by different factors, including central metabolism. It has been described that SucC and SucD, but not other TCA cycle enzymes, negatively affect β-lactam resistance driven by the penicillin-binding protein 2a. Mutations in the genes encoding these enzymes lead to changes in the succinyl-CoA level, globally changing the succinylation of proteins. One of the most affected proteins is the autolysin Atl and, as a consequence of these changes in its structure, the susceptibility to β-lactams, methicillin included, increases[87]. Further, it has been shown that the disruption of protein folding may improve antibiotic activity as well[88].

All of these observations indicate that antibiotic susceptibility and antibiotic resistance evolution are contingent on bacterial metabolism. Actually, it has been described that the susceptibility to antibiotics can be under the control of global metabolic regulators[89] and that the mutation of core metabolic genes provides clinically relevant antibiotic resistance[62]. Consequently, global analyses of metabolic changes by using metabolic models of bacteria exposed to antibiotics[90] or of antibiotic-resistant organisms[91,92] could help to identify targets and compounds that specifically act on resistant bacteria[91]. Further, understanding the changes of bacterial metabolism in response to antibiotics may suggest alternative and less conventional therapeutic strategies to reduce the probability of the emergence and evolution of antibiotic resistance[93].

Ancestral role of resistance determinants

The threat that antimicrobial resistance constitutes to human health has led to an intensive study of the role and relevance of bacterial antibiotic resistance determinants in clinics. However, these elements may have other ancestral functions besides antibiotic resistance[94]. Indeed, some non-antibiotic-producing bacteria, such as *P. aeruginosa* or *Stenotrophomonas maltophilia*, which are common inhabitants of natural environments, have a low intrinsic susceptibility to different antimicrobials, owing to the activity of efflux pumps or of enzymes capable of modifying antibiotics[95]. The fact that antibiotic concentrations in natural ecosystems are usually low suggests that these determinants did not evolve to provide bacteria with resistance to currently used drugs. Antibiotic resistance could thus be a novel role of these determinants that has emerged as a result of the use of antibiotics[96,97]. In fact, this is far from surprising if we understand evolution as a process that uses the pieces already available to find an optimal solution to a new challenge, such as the massive use of antibiotics by humankind.

Regarding antibiotic-modifying enzymes, AmpC β-lactamase-inactivating and aminoglycoside-inactivating enzymes are widespread intrinsic resistance elements. For the latter, a role of *Providencia stuartii* gentamycin 2′-*N*-acetyltransferase in peptidoglycan recycling has been demonstrated. This enzyme is a peptidoglycan *O*-acetyltransferase that inactivates gentamycin because the antibiotic has similarities with its natural substrate[98]. Although not formally demonstrated, a function of AmpC β-lactamases in peptidoglycan remodelling has been suggested. Supporting this hypothesis, it has been shown that AmpC is involved in *E. coli* morphogenesis[99] and that its expression is regulated by the cell morphology regulator BolA[100]. The expression of *P. aeruginosa* AmpC and the genes involved in the peptidoglycan recycling pathway present a complex cross-regulation, and therefore a better understanding of this cross-regulation may aid in the development of more efficient β-lactams[101]. Although less widespread, the fosfomycin-inactivating enzyme FosA is an intrinsic resistance determinant in some bacteria, such as *P. aeruginosa*. Although its original substrate is unknown, it belongs to the family of glutathione transferases[102], which are regularly involved in detoxification and biodegradation processes[103]. Finally, it has also been shown that the quinolone resistance gene *qnrA* is involved in the cold-shock response in its original host, *Shewanella algae*[104].

The role of efflux pumps, which are among the most relevant antibiotic resistance determinants, in different processes of bacterial physiology has been explored in some detail. Efflux pumps are not only involved in the extrusion of antibiotics outside the cell but also detoxify other categories of exogenous compounds such as heavy metals, biocides, organic pollutants or host-produced compounds[105]. In addition, efflux pumps can also extrude endogenous metabolites, including quorum-sensing signals and their precursors, which regulate bacterial virulence. For instance, the MexAB–OprM, MexCD–OprJ and MexEF–OprN *P. aeruginosa* efflux pumps extrude quorum-sensing-related molecules and also several types of antimicrobials. Hence, mutants overproducing these systems are resistant to different drugs and they simultaneously exhibit a modified virulence potential. The involvement of efflux pumps in various physiological processes, including detoxification of metabolites or agents from the environment, modulation of quorum sensing (eventually influencing virulence) or regulation of host–bacteria interactions, suggests that their ancestral functions differ from their recently acquired role in the extrusion of clinically used drugs[105].

Learning about the ancestral role of these antibiotic resistance determinants might be useful for designing strategies to tackle bacterial infections. For instance, efflux pumps of plant-infecting microorganisms can detoxify plant-produced flavonoids that inhibit bacterial virulence, therefore facilitating the colonization of plant tissues[106,107]. In addition, such plant-derived substrates induce the expression of the genes encoding these efflux pumps, which increases their extrusion, indicating that these determinants have an ancestral role in plant–bacterial interactions. Notably, this role of efflux pumps has been observed in bacteria that cause human infections: the major quinolone resistance determinant in the opportunistic pathogen *S. maltophilia*, the SmeDEF efflux pump, was shown to be involved in the colonization of plant roots[107]. This suggests that efflux pumps of opportunistic pathogens with environmental origin might interact with plant-produced compounds that are able to reduce bacterial virulence. Following such a hypothesis, several compounds that reduce the virulence of *P. aeruginosa* have been found by searching for molecules that are both substrates and inducers of the same efflux pump[12]. Although these anti-virulence compounds are inducers of the expression of efflux pumps, they do not always increase antibiotic resistance (Fig. 5). This would be the case for efflux pumps that extrude both the antibiotic and the inducer because, despite the efflux pump being induced, both compounds compete for extrusion. Therefore, this type of anti-virulence molecule, found by considering the original role of efflux pumps, might be used in clinics — alone or in combination with antibiotics — to counteract *P. aeruginosa* infections. Further, a more straightforward approach to find inhibitors of efflux pumps has been proposed taking into consideration the function of the *Neisseria gonorrhoeae* MtrCDE efflux pump. This antibiotic resistance determinant extrudes, besides antibiotics, host-produced bile salts and antimicrobial peptides, hence being an important virulence determinant. Knowing this function and its capacity to extrude antimicrobial peptides, a peptide has been designed with the ability to increase the antibiotic susceptibility of different rod-shaped Gram-negative bacteria by binding within the periplasmic region of RND-type efflux pumps[108].

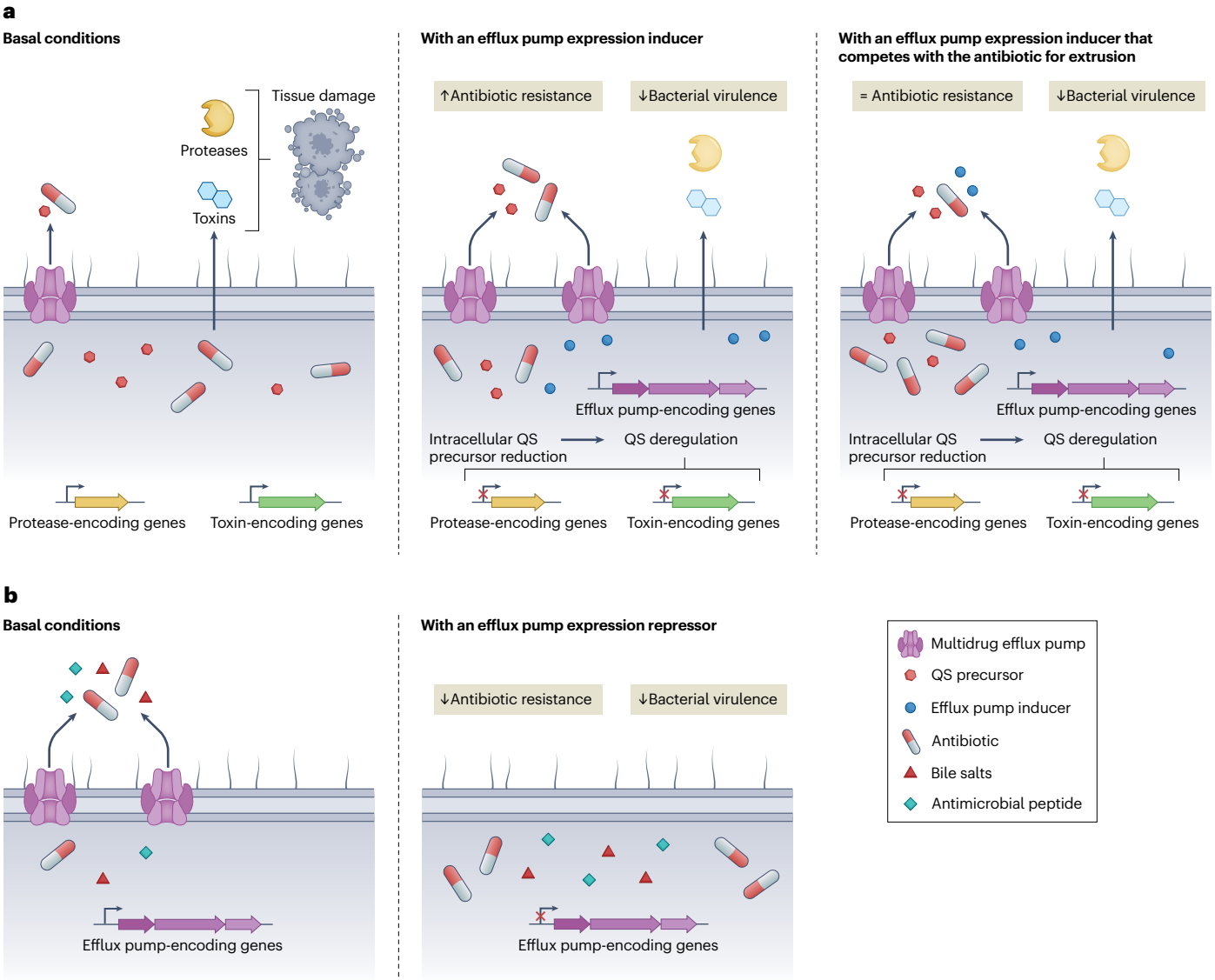


Fig. 5 | Interfering with the activity of efflux pumps to impair bacterial virulence. Besides contributing to antibiotic resistance, efflux pumps may be important elements for bacterial virulence. Depending on the organism and the efflux pump, two different situations have been reported. **a**, Overexpression of efflux pumps reduces bacterial virulence. This was, for instance, shown in *Pseudomonas aeruginosa*, in which virulence declines owing to the extrusion of quorum-sensing (QS) signals or their precursors[150] (left). In this case, inducers of efflux pumps will reduce bacterial virulence, but will also increase antibiotic resistance (middle) unless they are also substrates able to compete with the antibiotics for their extrusion from the cell (right). Some plant-produced compounds, which are likely to have anti-virulence properties in the field, have been identified as adjuvants that impair *P. aeruginosa* virulence without increasing antibiotic resistance[12]. **b**, Efflux pumps (and their overproduction) are needed for the virulence of the pathogen. This is the case for the AcrAB efflux pump from *Salmonella* spp. (and from other Enterobacteriaceae) or the *Neisseria gonorrhoeae* MtrCDE efflux pump, which extrudes bile salts and antimicrobial peptides as well as antibiotics[151,152] (left). In this case, the inhibition of the efflux pump will simultaneously reduce antibiotic resistance and bacterial virulence (right).

Given their ubiquity, efflux pumps constitute the antibiotic resistance determinants whose roles, beyond resistance, have been explored in more detail. Nevertheless, for different antibiotic resistance determinants, other functions beyond antibiotic resistance, including pathogenicity, have also been described, such as the aforementioned *L. monocytogenes* Hpt transporter (see above) or some antibiotic-modifying enzymes. For instance, the *Mycobacterium* enhanced intracellular survival (Eis) protein is an acetyltransferase that acetylates different aminoglycoside antibiotics[109]. This acetyltransferase also acetylates some proteins, such as the bacterial nucleoid-associated protein HU, which has a role in bacterial DNA compaction, or the human histone H3, which regulates the production of IL-10 (refs. 109,110). Therefore, this enzyme, in addition to contributing to antibiotic resistance, has a central role in *Mycobacterium* physiology and pathogenicity. Studying these protein functions may lead to the discovery of the conditions or compounds that reduce protein activity, thus reducing

both aminoglycoside resistance and *Mycobacterium* virulence. It is worth mentioning that the inhibitors of Eis include metal ions such as Au^{3+}, Zn^{2+} and Cd^{2+} (ref. 111). Among these, Zn^{2+} salts, such as zinc pyrithione, are the least toxic in a rat model and thus the most relevant for potential application[111].

Eco-evolutionary based approaches

Evolution can only be understood within the frame of ecology. The reason is that, although genes are expressed in individual organisms, they also modify the structure of microbial populations and communities in various environments. Thus, ecology-based approaches are also of utmost relevance in the effort to impair antibiotic resistance evolution. Studying prey–predator relationships, clonal interference, interbacterial competition (and even plasmid competition) or cross-feeding, and understanding how these situations affect and are affected by the acquisition of antibiotic resistance, may help in developing ecology-based approaches to tackle this issue. Among them, faecal microbiota transplantation (FMT) and the use of probiotics or bacterivorous predators to displace or eradicate antibiotic-resistant populations are among the most promising.

The human gut is a complex microbial ecosystem of symbiotic gastrointestinal bacteria. Novel methods of cultivation and sequencing analysis are improving our understanding of the components of the gut microbiome[112–114]. Dysbiosis, including dysbiosis due to antibiotic use, can favour colonization by non-commensal pathogens as well as by MDR microorganisms, and can also provide a suitable environment for the horizontal gene transfer of antibiotic resistance genes (ARGs)[115,116]. One of the strategies to deal with intestinal infection and colonization by antibiotic-resistant bacteria is the aforementioned FMT, a way to restore the human gut microbiome by transferring the microbiota from healthy donors into the colon or upper small intestine of patients[117]. Although it has been widely proved that FMT can reduce ARGs in patients infected with *Clostridium difficile*[118], studies on its safety and its effect on other MDR bacteria are still ongoing. Several studies have hinted that this approach, alone or in combination with antibiotic treatment, is effective in eliminating intestinal colonization by carbapenemase-producing, extended-spectrum β-lactamase-producing Enterobacteriaceae[119,120], as well as in decreasing the number and expression levels of ARGs in the microbiome. However, the risks and long-term consequences of microbiota manipulation[121] make a pre-transplantation analysis of the microbiome of both donor and patient mandatory, which potentially enables the design of cocktails of probiotic microorganisms that would efficiently compete with MDR bacteria. Among the potential benefits of this approach, probiotics can decrease *Helicobacter pylori* colonization by restoring the mucosal barrier or inhibiting the adherence of the pathogen, among other effects[122]. This approach may reduce the need to use antibiotics against *H. pylori*, hence reducing the chances of selecting antibiotic resistance. Besides displacing pathogens, probiotics can interfere with the transfer of conjugative plasmids, thereby impairing antibiotic resistance transmission[123]. In addition, it has been shown that the introduction of a probiotic harbouring a broad-range self-transmissible plasmid leads to the displacement of antibiotic resistance plasmids in a microbiome of a mouse model[124]. Further, the use of a mixture of probiotics in vivo is associated with a decrease in the colonization of the intestinal microbiota by β-lactamase AmpC-producing Enterobacteriaceae[125]. The combined use of antibiotics and probiotics is another emerging strategy to combat MDR bacteria[126]. Nevertheless, the probiotics used in this case must be resistant to the antibiotics administered in combination, a feature of concern when resistance is due to the presence of plasmids harbouring ARGs[127]. Further, although it seems to be a very infrequent event, sepsis due to the use of probiotics has been reported in extremely preterm infants[128]. Thus, although promising, the use of FMT and of probiotics to tackle antibiotic resistance still requires further investigation to be fully implemented in clinical practice[129].

Another ecology-based approach that deserves mention is the use of bacterivorous predators. Among them, *Bdellovibrio bacteriovorus* stands out because of its ability to prey on several other bacteria, including drug-resistant ones, such as *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *S. maltophilia*, *E. coli* or *S. aureus*[130,131]. However, it is worth mentioning that predation is strain specific, a feature that may hinder the applicability of these findings[132]. Multiple studies have shown that the predatory bacteria *Bdellovibrio* and other *Bdellovibrio*-and-like organisms[133], such as *Micavibrio aeruginosavorus*, could be used as therapeutic agents to tackle antibiotic resistance, either by killing the MDR bacteria[134] or by degrading ARGs present in the environment[135]. In addition, these organisms could be used in combination with antimicrobials to achieve the complete annihilation of the targeted pathogen[136], although this requires investigating the ability of *Bdellovibrio*-and-like organisms to resist antibiotics themselves[137]. Alternatively, these predator–antibiotic combination therapies could also be exploited using amoebae, which are not inhibited by antibiotics, together with broad-spectrum antimicrobials[138].

It is worth pointing out that medicinal larvae have been approved for use in humans and animals infected by antibiotic-resistant organisms[139]. However, although these ecology-based courses of action seem quite promising, their application demands further research because uncontrolled administration of predators might damage the microbiome[130,140]. This possibility supports the need for predators that specifically eliminate pathogens, particularly the antibiotic-resistant ones, an aspect still underexplored in the study of these prey–predator interactions. Of note, several studies using *Caenorhabditis elegans* as a virulence model show that, although wild-type antibiotic-susceptible pathogens kill this bacterivorous nematode, the worm can grow on antibiotic-resistant mutants[141]. It is then possible that some bacterivorous organisms could more efficiently eliminate resistant bacteria than wild-type ones. Identifying those bacterivorous organisms that efficiently and specifically eliminate antibiotic-resistant bacteria will help to counteract antibiotic resistance evolution. In this regard, we point out that these organisms could be used not just in the clinic, but also in other reservoirs relevant to antibiotic resistance transmission, such as wastewater[3].

Finally, we should also take into consideration that bacteria are part of complex ecological environments; therefore, targeting host–microorganism and bacteria–bacteria interactions and comprehending competition, symbiosis, mutualism and community organization could help in counteracting antibiotic resistance[142]. An example of how competition affects antibiotic resistance is the interplay between conjugative MDR plasmids and the constitutively active type VI secretion system (T6SS) of *A. baumannii*. T6SS serves to kill non-kin bacteria; hence, its expression in plasmid donors or recipients may impair conjugation. Notably, MDR plasmids repress T6SS in their hosts, which enables their spread[143]. Another evolutionary adaptation is mutualism, and the clearest example is the formation of biofilms, in which the transfer of ARGs is favoured[144]. Regarding community organization, it has been described that chromosomal *S. maltophilia* metallo-β-lactamases can protect *P. aeruginosa* from imipenem action when these species

co-infect patients with cystic fibrosis[145]. It has also been described that extrusion of antimicrobial peptides, as microcins, in the intestine favours increases in the population size and spread of MDR clones of *E. coli*[72].

In summary, all these data suggest that ecology-based features should be taken into account when designing alternative approaches to curb antibiotic resistance evolution. Among them, it has been proposed that cross-feeding[146,147] or competitive interactions among bacteria or, eventually, among mobile genetic elements[124,148,149] could be exploited to tackle antibiotic resistance. It is important to note that most of those examples are based on experimental, laboratory-adapted model strains, so exploring these and other ecology-based applications to tackle antibiotic resistance evolution using clinical isolates will be crucial in the near future.

Concluding remarks

Most efforts to control the rise of antibiotic resistance have focused on decreasing its emergence by reducing selective pressure (antibiotic use) and resistance transmission[3]. The control of resistant bacteria has been traditionally addressed through the development of novel antimicrobials; however, in a situation in which the antibiotic pipeline is nearly empty, other approaches are needed. Among them, an improved use of existing antibiotics seems promising. The search for adjuvants or the identification of simultaneous or sequential antibiotic combinations is usually performed by means of blind, not evidence-based, assays. Nevertheless, knowing the effect of antibiotic resistance evolution on bacterial physiology, including metabolic alterations and changes in the susceptibility to other drugs (collateral sensitivity), can guide the identification of more effective compounds and antibiotic combinations that can exploit the bacterial weaknesses that emerge as a consequence of bacterial drug resistance evolution. One important, and frequently underexplored, feature for such exploitation to be feasible is phenotypic robustness, understood as the conservation of the exploitable phenotype in different bacteria and under different conditions. The identification of robust patterns of collateral sensitivity and of re-sensitization in the absence of drugs, as well as of metabolic changes associated with resistance acquisition, together with the modulation of the ecological landscape of pathogens, is providing valuable information for better use of current antibiotics and better management of antibiotic resistance.

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The authors declare no competing interests.

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