Antibiotic resistance and its cost: is it possible to reverse resistance?

Dan I. Andersson and Diarmaid Hughes‡*

Abstract | Most antibiotic resistance mechanisms are associated with a fitness cost that is typically observed as a reduced bacterial growth rate. The magnitude of this cost is the main biological parameter that influences the rate of development of resistance, the stability of the resistance and the rate at which the resistance might decrease if antibiotic use were reduced. These findings suggest that the fitness costs of resistance will allow susceptible bacteria to outcompete resistant bacteria if the selective pressure from antibiotics is reduced. Unfortunately, the available data suggest that the rate of reversibility will be slow at the community level. Here, we review the factors that influence the fitness costs of antibiotic resistance, the ways by which bacteria can reduce these costs and the possibility of exploiting them.

Fitness
The capability of a genotype or individual to survive and reproduce.

Bypass resistance
The replacement (bypass) of a metabolic step that is normally inhibited by an antibiotic with a new, drug-resistant metabolic enzyme.

**Department of Medical Biochemistry and Microbiology, Uppsala University, BOX 582, SE-751 23, Uppsala, Sweden. ‡Department of Cell and Molecular Biology, Uppsala University, BOX 596, SE-751 24, Uppsala, Sweden. Correspondence to D.I.A. e-mail: dan.andersson@imbim.uu.se*
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The introduction of antibiotics is one of the most important medical interventions with regard to reducing human morbidity and mortality. However, the intensive use of antibiotics (which was estimated in 2002 to be 100,000–200,000 tonnes per annum worldwide[1] and which is, in total, well over 1 million tonnes since the 1940s) has dramatically increased the frequency of resistance among human pathogens and threatens a loss of therapeutic options and a post-antibiotic era in which the medical advances to date are negated[2–4]. Resistance dramatically reduces the possibility of treating infections effectively and increases the risk of complications and of a fatal outcome.

Resistance is often associated with reduced bacterial fitness, and it has been proposed that a reduction in antibiotic use (and, therefore, in the selective pressure to acquire resistance) would benefit the fitter susceptible bacteria, enabling them to outcompete resistant strains over time[5,6]. Experimental studies and theoretical modelling support this basic concept[5,7], but other processes, such as compensatory evolution and genetic co-selection, complicate the picture and make reversibility less probable in real-life settings. This Review focuses on experimental studies of the factors (in particular, the fitness costs of resistance) that influence the development of resistance and the potential for reversibility. We describe the outcomes of laboratory studies and clinical interventions to reduce the frequency of resistant bacteria in individual patients as well as at the community level and explain why reversibility, if it occurs at all, proceeds so slowly that, in most cases, it is unlikely to be of practical importance.

The development of resistance and its reversibility

The rate of appearance of resistant bacteria. The rate of appearance of antibiotic-resistant bacteria is determined by the combined rates of *de novo* mutation and horizontal gene transfer (HGT) of resistance determinants (FIG. 1). The most common mutations lead to alterations in the antibiotic target and increases in drug efflux[8], but resistance is also associated with gene amplification[9,10], reduced expression of the target[11] and alteration of drug modification enzymes[12]. Mechanisms associated with HGT include drug modification, target protection, bypass resistance, replacement of susceptible drug targets and acquisition of novel efflux pumps[13]. Rates of mutation can be measured for most clinical pathogens, but there is very little information for HGT on which to base predictions regarding the sources of resistance genes[14] or the rates of transfer of these genes through the gene pool. This makes it difficult to predict how HGT will influence the emergence of resistance to novel drugs[15].

Selection of resistant bacteria. Resistance becomes a clinical problem when the frequency of the resistant variant threatens the effectiveness of empirical drug therapy. The level of exposure of the pathogen population to the drug influences the selection of resistant variants[16,17]. The exposure level is associated with pharmacokinetic and pharmacodynamic properties of the drug, which affect the clearance of the pathogen or the selection of resistant variants in the patient. It is also associated with hygienic measures and transmission control in clinics and hospitals and with the volume and distribution of antibiotics

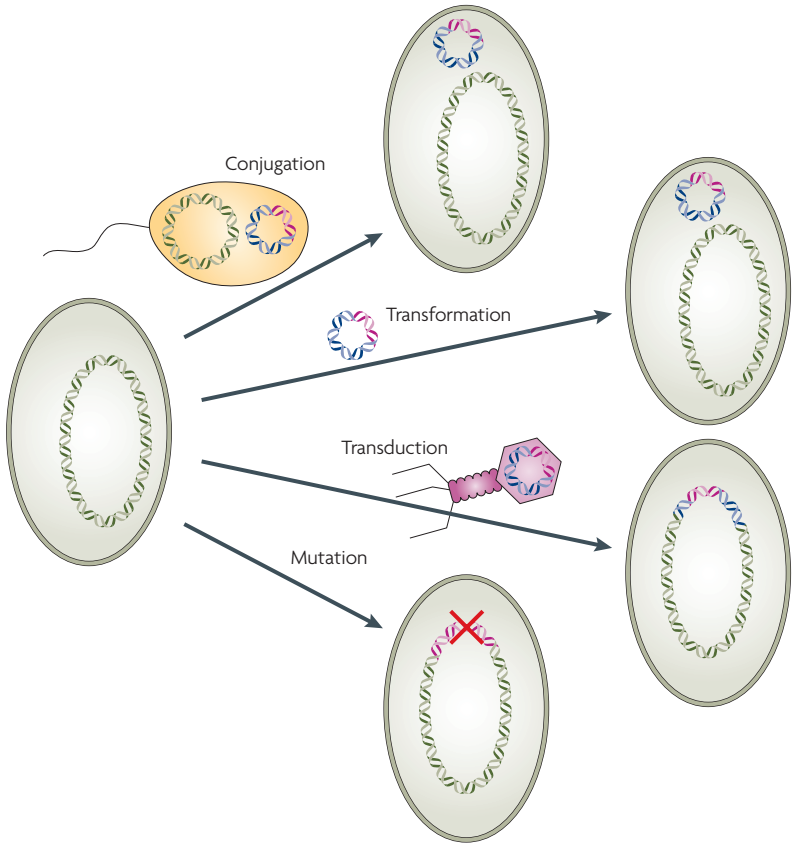

Figure 1 | **Mechanisms of resistance acquisition.** DNA from the biosphere containing an antibiotic resistance gene (pink) can be transferred by horizontal gene transfer into a recipient by several paths: cell-to-cell conjugation; transformation by naked DNA (on plasmids or as linear DNA) that is released by dead cells; or phage-mediated transduction. Resistance can also arise by *de novo* mutation (indicated by a red cross).

that are released into the environment, where they may select for resistance and the transfer of resistance by HGT. The threat from antibiotics that are released into the environment is illustrated by the existence of a large reservoir of resistance genes in the human microflora that could potentially serve as donors for the transfer of resistance to human pathogens[18].

Biological costs of resistance and compensatory evolution. Antibiotics target important functions such as cell wall synthesis, regulation of chromosome supercoiling, RNA transcription and protein synthesis, so it is not surprising that resistant mutants usually suffer a decrease in biological fitness (TABLE 1). The acquisition of resistance genes by HGT also carries a fitness cost[19,20]. The association of resistance with decreased fitness has fuelled the hope that a reduction in the use of antibiotics would lead to a reduction in the frequency of resistant bacteria, through natural selection. However, resistant bacteria can ameliorate the costs of resistance by acquiring additional fitness-compensatory mutations (TABLE 2).

Co-selection phenomena. Co-selection of resistance to more than one antibiotic, owing to genetic linkage of the resistance genes, is a common feature of resistance acquired by HGT. The frequency of resistance to an antibiotic may rise, even if that antibiotic is not currently in use. For example, reducing the use of sulphonamides had no effect on the frequency of sulfonamide resistance in *Escherichia coli*[21], possibly because the plasmid-borne sulphonamide resistance gene is genetically linked to other resistance genes that continued to be selected during the study period. A recent example in which trimethoprim use was reduced in a Swedish county is discussed below. In principle, co-selection could also drive up the frequency of a linked drug-susceptible allele, leading to clonal replacement with this allele throughout the population. Co-selection also occurs when resistance to one drug confers increased resistance to drugs with a similar structure. This is especially problematic if different variants of a drug are used in agriculture and in medicine, with different regulatory controls. The development of resistance in one environment may drive the frequency of resistance in another environment.

The magnitude of fitness costs that is required for reversion of resistance. The time required to reduce the abundance of resistant bacteria is inversely related to the cost of resistance[22,23] (BOX 1). In one experimental study, the loss of plasmid-encoded tetracycline resistance from *E. coli* was measured over 500 generations[24]. The best-fit mathematical model included a high mutation frequency (~10^{-4} mutations per generation) for the loss of tetracycline resistance by deletion combined with a small but statistically significant selection coefficient ($s = 0.007$) associated with carriage of tetracycline resistance. In this case, $s = 0.007$ corresponds to a 0.7% reduction in growth rate. With these parameters, replacement of 99.9% of the population by susceptible bacteria would take approximately 1.5 years. To reach the same replacement level in 5 weeks, the selection coefficient associated with resistance would need to be at least 0.06, which is realistic for a constitutively expressed tetracycline resistance operon on a high-copy-number plasmid but not for an induced resistance gene on a low-copy-number plasmid with a broad host range[24]. This experiment supports theoretical arguments that in community settings the cost of resistance is the key factor to successful displacement of antibiotic-resistant populations with antibiotic-sensitive ones[23]. However, if even a tiny fraction of resistant bacteria remain in the population, the reintroduction of the antibiotic is predicted to cause the resistant bacteria to reach high frequencies again, at a much faster pace than the original decline[24].

Fitness costs and compensatory evolution

Establishing the connections between resistance and fitness requires experiments on isogenic strains (FIG. 2). This generates an experimental basis on which to interpret the phenotypes of clinical isolates that are usually of uncertain origin. In rare cases, isogenic clinical isolates that differ only in their resistance and fitness phenotypes have been isolated from the same patient[25] or shown by molecular analysis to be directly related[26,27]. Analysing such isolates can test the robustness of the models that are built using laboratory strains.

Pharmacokinetic properties
Characteristics of a drug that include: its mechanisms of absorption and distribution; the rate at which its action begins and the duration of the effect; the chemical changes of the agent in the body; and the effects and routes of excretion of drug metabolites. Often summarized as what the body does to a drug.

Pharmacodynamic properties
Characteristics of a drug that include: the physiological effects of a drug on the body, on microorganisms or on parasites in or on the body; the mechanisms of drug action; and the relationship between drug concentration and effect. Often summarized as what a drug does to the body.

Table 1 | **The biological cost of antibiotic resistance conferred by chromosomal mutations**

Bacteria	Resistance	Cost*	Assay system	Refs
Salmonella enterica subsp. *enterica* serovar Typhimurium	Streptomycin	Variable	Mice and *in vitro*	29,31,88,91
	Rifampicin	Variable	Mice and *in vitro*	29,49
	Nalidixic acid	Yes	Mice and *in vitro*	29
	Ciprofloxacin	Yes	Chickens and *in vitro*	124
	Fusidic acid	Variable	Mice and *in vitro*	40–42,92
	Mupirocin	Yes	Mice, nematodes and *in vitro*	125
	Actinonin	Yes	Mice, nematodes and *in vitro*	96
Escherichia coli	Streptomycin	Variable	*In vitro*	126,127
	Norfloxacin	Variable	Mice and *in vitro*	58
	Rifampicin	Variable	*In vitro*	46
	Fosfomycin	Yes	Urine and *in vitro*	128
Campylobacter jejuni	Ciprofloxacin	Variable	Chickens	34
Mycobacterium tuberculosis	Isoniazid	Yes	Mice	73,99
	Rifampicin	Yes	Macrophages and *in vitro*	26,129,130
Mycobacterium bovis	Isoniazid	Yes	Mice	72
Mycobacterium smegmatis	Streptomycin	Variable	*In vitro*	30
Staphylococcus aureus	Fusidic acid	Variable	Rats and *in vitro*	39,131,132
	Rifampicin	Variable	Biofilms and *in vitro*	45,47,133
	Mupiricin	No	Mice and *in vitro*	33,134
	Methicillin	Yes	*In vitro*	32
	Vancomycin	Variable	*In vitro*	57
Staphylococcus epidermidis	Fusidic acid	Yes	Humans	135
	Ciprofloxacin	No	Humans	135
Streptococcus pneumoniae	Gemifloxacin	Yes	Mice and *in vitro*	136
Helicobacter pylori	Clarithromycin	Yes	Mice and *in vitro*	25,27
Chlamydia psittaci	Spectinomycin	Yes	*In vitro*	137
Pseudomonas aeruginosa	Fluoroquinolone	Variable	*In vitro*	82,138
Pseudomonas fluorescens	Rifampicin	Yes	Soil	139
Listeria monocytogenes	Class IIa bacteriocin	Yes	*In vitro*	140
Neisseria meningitidis	Sulfonamide	Yes	*In vitro*	141

*Yes indicates that the increase in the generation time, which is a measure of the fitness cost, ranges from several percent up to as much as 400%. Variable means that some mutations have an associated fitness cost, whereas others do not.

Fitness is relative and should be measured in different environments[28,29] to establish its predictive value in determining the clinical outcome of infection. In some cases, resistant mutants appear to be as fit as the wild-type bacteria[29,30], raising the question of how to interpret negative results[31]. However, if fitness costs are observed in laboratory experiments, it is reasonable to assume that there will also be clinical conditions under which the resistance would impose a fitness burden.

Fitness costs of resistance measured* in vitro *and in animals. The fitness costs of resistance can be considerable (TABLE 1). There is no obvious correlation between the magnitude of the cost and the type of target molecule inhibited by the antibiotic, although an inverse relationship between the resistance level of an isolate and its growth rate is observed in some exceptional cases[32,33]. Below, we outline five lessons that have emerged from studies of fitness costs.

The first lesson is that epistasis can affect fitness costs. The fitness costs of streptomycin resistance caused by mutations in *rpsL* (which encodes 30S ribosomal protein S12) depend on environmental and epistatic effects. In *Salmonella enterica* subsp. *enterica* serovar Typhimurium, streptomycin-resistant (Sm^R) mutants with K42N or P90S substitutions have impaired growth on rich medium but, surprisingly, grow faster than wild-type bacteria in media with poorer carbon sources[31]. The increased growth rate reflects a failure of these mutants to induce the stress-inducible RNA polymerase σ-factor (σ^S; also known as RpoS), which is a key regulator of many stationary-phase and stress-inducible genes;

Selection coefficient
A measure of the fitness of a phenotype relative to wild type (often denoted *s*), having a value between 0 and 1. When $s = 0$, there is no fitness reduction, and when $s = 1$, the mutation is lethal.

Epistasis
An interaction between genes such that the effect of one gene is modified by one or several other genes.

Table 2 | **Amelioration of the fitness costs that are caused by chromosomal mutations**

Bacteria	Resistance mutation	Resistance to	Compensatory mutation	Resistance in compensated mutant	Selection system	Refs
Salmonella enterica subsp. *enterica* serovar Typhimurium	*rpsL*	Streptomycin	Intragenic	Maintained	Mice	29
	rpsL	Streptomycin	Extragenic (*rpsD* or *rpsE*)	Maintained	Laboratory medium	29,91
	gyrA	Nalidixic acid	Intragenic	Maintained	Mice	29
	rpoB	Rifampicin	Intragenic	Maintained	Mice	29
	fusA	Fusidic acid	True reversion	Lost	Mice	28
	fusA	Fusidic acid	Intragenic	Often maintained	Laboratory medium	92
	fmt and *folD*	Actinonin	Intragenic	Maintained	Laboratory medium	96
	fmt	Actinonin	Extragenic (*metZ* and *metW* amplification)	Maintained	Laboratory medium	96
Escherichia coli	*gyrA* and *marR*	Fluoroquinolones	Extragenic (*parC*)	Increased	Constructed* or laboratory medium	62
	gyrA, *parC* and *marR*	Fluoroquinolones	*gyrA*-2	Increased	Constructed*	62
	rpsL	Streptomycin	Extragenic (*rpsD* or *rpsE*)	Maintained	Laboratory medium	126,127
	rpoB	Rifampicin	Intragenic	Maintained	Laboratory medium	46
Staphylococcus aureus	*fus*	Fusidic acid	Intragenic	Maintained or lost	Laboratory medium	39
	rrl	Linezolid	Gene conversion	Lost	Laboratory medium	93,94
Streptocccus pneumoniae	*gyrA*, *parC* and *parE*	Fluoroquinolones	Intragenic	Maintained or increased	Constructed*	97
Mycobacterium tuberculosis	*katG*	Isoniazid	Extragenic (*ahpC*)	Maintained	Humans	101
Neisseria meningitidis	*sul2*	Sulfonamide	Intragenic	Maintained	Humans	141

ahpC, alkyl hydroperoxide reductase C gene; *fmt*, methionyl-tRNA formyltransferase gene; *fusA*, elongation factor G gene; *gyrA*, DNA gyrase subunit A gene; *katG*, catalase–peroxidase gene; *marR*, multiple drug resistance protein R gene; *met*, methionine biosynthesis; *parC*, DNA topoisomerase IV subunit A gene; *parE*, DNA topoisomerase IV subunit B gene; *rpoB*, DNA-directed RNA polymerase subunit-β gene; *rpsD*, 30S ribosomal protein S4 gene; *rpsE*, 30S ribosomal protein S5 gene; *rpsL*, 30S ribosomal protein S12 gene; *rrl*, 23S ribosomal RNA gene; *sul2*, dihydrofolate synthase 2 gene.*Mutants were constructed rather than selected for.

the precise mechanism linking the ribosomal gene mutation with reduced σ^S levels has not yet been elucidated. On poorer carbon sources, wild-type cells induce σ^S, thus slowing their growth. By not inducing σ^S, the Sm^R mutants escape this self-imposed growth inhibition. The results suggest that the induction of σ^S contributes to long-term cell survival, even though it actually limits the short-term growth rate under restrictive growth conditions. These results also highlight the importance of measuring fitness costs under multiple experimental conditions, not only to acquire a more relevant estimate of fitness, but also to reveal physiological weaknesses that may be exploitable for drug development[31]. Another example of an epistatic effect on fitness costs is shown by a mutation in the gene encoding DNA gyrase subunit A (*gyrA*) that causes resistance to ciprofloxacin (a fluoroquinolone) in *Campylobacter jejuni*. In the absence of antibiotic selection, this *gyrA* mutation enhanced the fitness of the resistant strain when it was in competition in a chicken infection model[34]. However, the same mutation imposed a fitness cost when it was transferred into a different genetic variant of *C. jejuni*. The surprising implication is that the rapid emergence of fluoroquinolone-resistant *Campylobacter* spp. may be due to the enhanced fitness that is associated with resistance.

Plasmids carrying drug resistance genes usually impose a fitness cost on the bacteria that harbour them, resulting in reduced growth rate[19,20,35–37]. One example is the plasmid carrying the gene that encodes AmpC-type β-lactamase, which is rarely found in clinical isolates of *Salmonella* spp.[38]. When *ampC* was experimentally transformed into *S.* Typhimurium, it reduced the growth rate and invasiveness of the bacterium, suggesting that the associated reduced fitness is a plausible reason for the rarity of the plasmid[38]. However, if both *ampC* and its regulator, *ampR*, were introduced, making β-lactam resistance inducible rather than constitutive, these fitness costs were eliminated[38]. Thus, the genetic context of the resistance gene can determine the fitness cost associated with its acquisition.

The second lesson is that environmental conditions affect fitness costs. Some resistance mutations that show no cost in bacteria grown in laboratory medium have high costs in laboratory mice and, conversely, some mutations that show no cost in mice can have substantial costs *in vitro*[28,39]. A mutation in *fusA*, the gene encoding elongation factor G (EFG), results in fusidic acid-resistant (Fus^R) *S.* Typhimurium by altering levels of the transcriptional regulator guanosine tetraphosphate (ppGpp)[40]; this mutation has pleiotropic effects

Box 1 | **Measuring fitness costs and compensation**

Single culture and competition experiments can be carried out *in vitro*, and exponential growth rates can be measured for many bacterial cultures in parallel using microtitre plate machines. It is possible to discriminate differences of ~5% in generation time.

Greater discrimination can be achieved in pairwise competitions[29,62]. Mixed cultures are serially passaged through cycles of growth and re-inoculation and assayed for changes in the ratio of the two strains. Growth cycles measure several components of competitive fitness, including lag periods, rates of exponential growth and efficiencies of resource utilization. Neutral genetic tags in one or both strains permit detection of fitness differences that are ≤ 1% per generation[137]. With FACS (fluorescence-activated cell sorting) analysis using fitness-neutral fluorescent markers[138], it may be possible to detect fitness differences of 0.1% per generation.

Single culture and pairwise competition experiments between susceptible and resistant bacteria can also be carried out in animal models, in which the complex growth environment has more relevance to the clinical infection, as rates of clearance and mortality can be measured in the presence or absence of host immune defences[41]. Bioluminescent measurements now permit tracking of tagged bacterial strains *in vivo* and provide real-time measurements of the colonization of different compartments and of competitive fitness[139–141].

on gene expression, possibly explaining the fitness differences that are observed in different environments[41,42]. Interestingly, the pleiotropic phenotypes of the *fusA* mutant also include hypersensitivity to unrelated antibiotics[43], which is a potentially exploitable 'Achilles heel' of resistance.

Rifampicin resistance is caused by mutations in DNA-directed RNA polymerase subunit-β (RpoB). All of the tested rifampicin-resistant (Rif^R) mutants have a reduced fitness[44–46], with the exception of the S464P mutant in *Staphylococcus aureus*, which was better adapted to growth in a mouse biofilm infection model than the susceptible parental strain[47]. Another intriguing observation with mutations that confer resistance to rifampicin is that they accumulate to very high frequencies in bacterial colonies that have been allowed to age on solid media. Although this was initially suggested to be the result of 'stress-induced' mutagenesis[48] in an ageing colony, it seems that, in fact, most pre-existing Rif^R mutants continue to grow, whereas normal cells remain quiescent. Thus, Rif^R mutants that have a reduced fitness in exponential growth have a notable growth advantage in the environment of the ageing colony[49]. This illustrates that fitness is strongly dependent on the environment in which it is measured.

The third lesson is that some mutations are cost free. Resistance to streptomycin that is caused by the K42R substitution in 30S ribosomal protein S12 is an apparent 'no-cost' mutation in *S.* Typhimurium and *E. coli*[50,51] (FIG. 3a). The mutation may even confer a slight advantage over the wild-type bacterium in mouse[29] and pig[52] infection models. This substitution is found at a high frequency in Sm^R clinical isolates of *Mycobacterium tuberculosis*[30,53]. This implies that the K42R mutation in 30S ribosomal protein S12 is a genuine cost-free resistance mutation.

The fourth lesson is that the cost of resistance can be reduced by regulation of the resistance mechanism. The VanA-type resistance to vancomycin/teichoplanin A-type glycopeptides in *S. aureus* is acquired by HGT of the resistance operon *vanA*[54]. Resistance results from the synthesis of alternative cell wall precursors (ending in D-alanyl–D-lactate) with very low affinity for glycopeptides and the elimination of the normal susceptible precursors (ending in D-alanyl–D-alanine) to which vancomycin binds[55]. The resistance phenotype is induced in response to the presence of glycopeptides[56]. Isogenic methicillin-resistant *S. aureus* strains with or without one of three different *vanA* resistance operons were identified in clinical isolates; pairwise comparisons of these isolates showed that when resistance was induced the fitness cost was considerable and growth rates were reduced by 20–38%[57]. By contrast, in the absence of induction the fitness cost of carrying the *vanA* operon was a 0.04–0.3% reduction in growth rate. Thus, VanA-type resistance is very costly for the methicillin-resistant *S. aureus* host when induced, whereas its biological cost is minimal in the absence of induction.

The fifth lesson is that cost compensation and resistance can be linked. Clinical resistance of *E. coli* to fluoroquinolones requires multiple genetic alterations involving mutation[58,59] and HGT[60,61]. Competition *in vitro* and in a mouse model showed that, on average, fitness decreased with the number of resistance mutations[62]. However, for some triple mutants the acquisition of a fourth resistance mutation increased fluoroquinolone resistance dramatically and also increased the fitness of the resistant strains[62]. Thus, in a particular genetic context a resistance mutation could increase both resistance and fitness, measured both *in vitro* and *in vivo*. This implies that Darwinian selection for improved fitness might, at least for the fluoroquinolones, drive the selection for increased drug resistance[62].

Costs of resistance estimated from clinical studies and epidemiology. By examining the resistance genes that can be found in bacteria isolated from humans, predictions from experimental studies of the costs of resistance can be tested. The K42R substitution in the 30S ribosomal protein S12, which showed no cost in *S.* Typhimurium and *Mycobacterium smegmatis* under experimental conditions[29], is also primarily responsible for resistance to streptomycin in clinical isolates of *M. tuberculosis*[30,53]. This suggests that no-cost (or low-cost) mutants are preferentially selected in humans. The spectrum of mutations in *rpoB* that cause rifampicin resistance in clinical isolates of *M. tuberculosis* and in *S. aureus* is also biased in favour of low-cost mutations, suggesting that fitness measured as growth rate *in vitro* is an important determinant of strain survival in the clinical environment[45,63].

Fusidic acid resistance in *S. aureus* has several different genetic causes, each of which has a different effect on fitness and is designated a resistance class. FusA-class resistance is caused by mutations in *fusA* (the gene that encodes the target of fusidic acid, EFG) and usually reduces fitness *in vivo* and *in vitro*[39,64]. By contrast, FusB-class and FusC-class resistance is conferred by the acquisition of *fusB* or *fusC*, respectively, which causes EFG to be protected from the effect of fusidic acid by another protein and has very low fitness costs[39,65]. The FusB and FusC

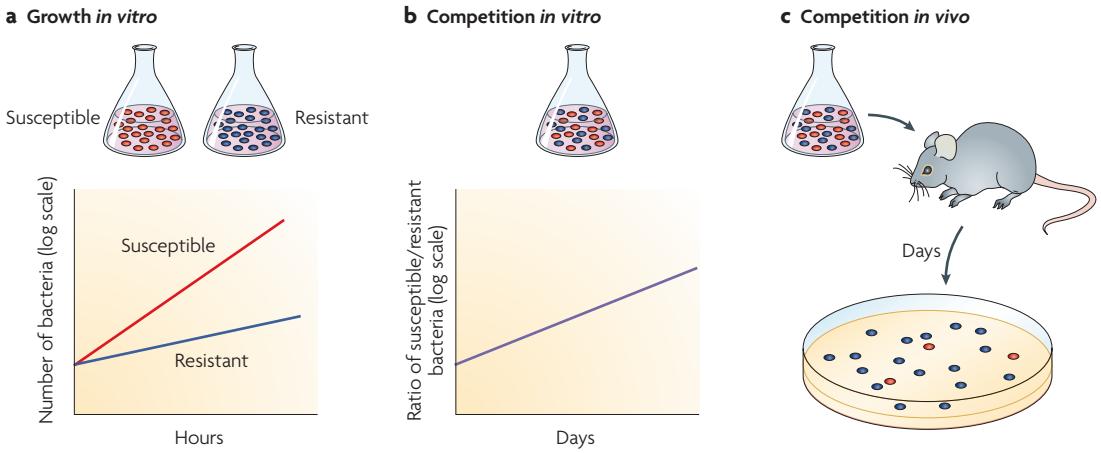

Figure 2 | **Determining fitness of bacterial strains. a** | Measuring exponential growth rates of susceptible and resistant strains *in vitro* can reveal large fitness differences between the strains. **b** | In an *in vitro* competition experiment in which cultures are cycled with a bottleneck each day, fitness differences between wild-type and mutant strains are revealed as a change in the ratio of the two competing strains. **c** | A suitable animal model can be infected with a mixed bacterial culture and assayed after several days to measure changes in the ratio of two populations.

resistance classes predominate in isolates from skin infections[66,67], whereas FusA, FusB and FusC resistance classes are equally prevalent in bacteraemia isolates[68]. Resistance caused by mutations in *rplF* (which encodes 30S ribosomal protein L6), designated FusE-class resistance, results in a variant that forms small colonies with an extremely slow growth rate[64]. Despite their extremely slow growth, these variants are found in clinical isolates of *S. aureus*[69], possibly because they can persist intracellularly, shielded from the host immune response[70]. Thus, the specific host environment could determine the fitness costs of different mechanisms of antibiotic resistance.

The impact of resistance on bacterial pathogenesis, virulence and disease pathology. Resistant mutants usually show decreased fitness as well as decreased virulence, even though there is no a priori reason that they should be correlated[71]. Strains of *Mycobacterium bovis* and *M. tuberculosis* that are resistant to isoniazid as a result of a T275P substitution in KatG (catalase–peroxidase) show severely reduced virulence as measured by host killing and by histopathology[72,73]. By contrast, strains of *M. tuberculosis* carrying the most common KatG mutation, an S315T substitution, are highly resistant to isoniazid and virulent in a mouse model of the disease[74,75].

Other examples in which disease pathology might be different between the resistant mutants and the wild-type bacteria are cephalosporin-resistant opportunistic Gram-negative bacteria such as *Citrobacter freundii* and *Enterobacter cloacae*, which encode an inducible chromosomal β-lactamase. Even with this resistance protein, the bacteria remain susceptible to third-generation cephalosporins. However, loss-of-function mutations in *ampD* (which encodes *N*-acetyl-anhydromuramyl-L-alanine-amidase and is required for peptidoglycan recycling) lead to constitutive β-lactamase production and resistance to third-generation cephalosporins[76]. As the mutation frequency during infection is high, *ampD* mutants arise during ongoing therapy, resulting in clinical failures[77–79]. These *ampD* mutants accumulate *N*-acetyl-anhydromuramyl-tripeptide in the cytoplasm[76] and, as a result, induce the production of nitric oxide in epithelial and phagocytic cells infected with *S. enterica*[80], which might alter the host inflammatory response. Mutations in the gene encoding multidrug resistance operon repressor, *mexR*, (known as the *nalB* mutation) and in *nfxB* that increase antibiotic resistance in *Pseudomonas aeruginosa* by upregulating drug efflux pumps increase the ability of the bacterium to make biofilms, which are relevant for the colonization of catheters and for persistence in chronic infections, but abolish its ability to kill *Caenorhabditis elegans*, which is used as a model for virulence[81,82]. The contrasting fitness effects of these efflux mutations suggests that one cannot necessarily infer general fitness defects *in vivo* by extrapolating from *in vitro* competition data.

Transmission rates for susceptible and resistant variants. For infectious bacteria to establish themselves within a host population, they must have a 'basic reproductive number' (R_0) of greater than 1 (REF. 83). R_0 is defined as the average number of secondary infections that are produced when an infected individual is introduced into a susceptible host population. An important question is whether transmission rates for susceptible and resistant bacteria differ greatly. Studies of *M. tuberculosis* transmission have produced contradictory results, with strains resistant to isoniazid showing reduced transmission[84] and Rif^R strains showing the opposite effect[85]. In addition, superspreading[86] (the phenomenon of a few individuals being responsible for the infection of unusually large numbers of secondary cases) can lead to heterogeneity in the R_0 of the infecting population and can influence the infection dynamics. More measurements of the transmission rates for resistant and susceptible variants that cause important infections are needed, and a recent study of *M. tuberculosis* shows how such data can be obtained from epidemiological studies[87].

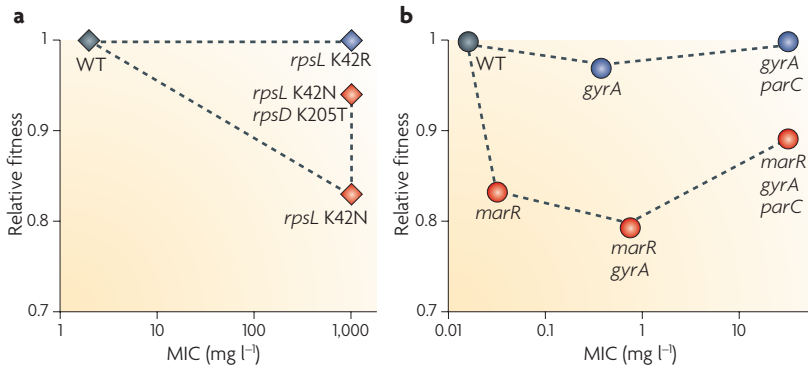

Figure 3 | **Relationship between antibiotic resistance and bacterial fitness.** Numbers shown on the *y* axes are from empirical data[28,61,88]. **a** | High-level resistance to streptomycin in *Salmonella enterica* subsp. *enterica* serovar Typhimurium (that is, strains with a minimal inhibitory concentration (MIC) of > 1,000 mg l^{-1}) can be cost free, as is the case for the resistance given by the mutation in *rpsL* (which encodes 30S ribosomal protein S12) that leads to a K42R substitution in the protein, or can reduce fitness, as seen for resistance imparted by a K42N substitution in the same protein. Reduced fitness can be compensated by extragenic mutations (for example, the mutation in *rpsD*, which encodes 30S ribosomal protein S4, that leads to a K205T substitution in the protein) without a loss of resistance[29,91]. **b** | Resistance to fluoroquinolones in *Escherichia coli* develops in multiple genetic steps. High-level resistance (that is, strains with an MIC for ciprofloxacin of > 32 mg l^{-1}) can develop without any substantial loss of fitness (as is the case for strains with mutations in the DNA gyrase subunit A gene, *gyrA*) or, following a different genetic trajectory, can involve a major reduction in fitness (as is the case for strains with mutations in the multiple drug resistance protein R gene, *marR*, or in both *marR* and *gyrA*). Note that resistance mutations in *marR*, which are knockout mutations, are intrinsically much more frequent than resistance mutations in *gyrA*, which are specific nucleotide substitutions. In the double mutant, acquisition of an additional fluoroquinolone resistance mutation in *parC* (which encodes DNA topoisomerase IV subunit A) can substantially compensate the fitness costs of resistance and, at the same time, greatly increase the level of resistance (the MIC in the double mutant is 0.75 mg l^{-1}, whereas in the triple mutant it is 32 mg l^{-1})[62]. In this figure, the *gyrA* mutation refers to the S83L, D87N double substitution.

Compensatory evolution can rapidly reduce the fitness cost of resistance. The decreased fitness caused by resistance may be rapidly and efficiently counterbalanced by compensatory mutations without a loss of resistance (FIG. 3; TABLE 2). Compensatory evolution can stabilize resistant bacterial populations in the absence of antibiotics by making them as fit as susceptible clones. Several *in vitro* and *in vivo* studies have shown that in most cases it is possible to detect compensation when resistant bacteria are serially passaged, but the degree of restoration and the type and number of compensatory mutations found vary depending on the particular resistance mechanism and bacterium as well as the environmental conditions under which compensation occurs (discussed below).

The selection of compensated mutants during serial passage depends on the mutation rates for the different types of mutations, the fitness of the different mutants and the bottlenecks during serial transfer[88–90]. Data from two studies using Fus^R *fusA* mutants or Sm^R *rpsL* mutants of *S.* Typhimurium suggest that the target size for compensatory mutations is typically more than 20 times the size of that for reversion and that population bottlenecks and growth conditions have strong effects on the spectra of compensatory mutations[28,29,39,91]. Thus, compensation is more likely than reversion (FIG. 3), as would be expected for many natural populations, in which bottlenecks are present. Compensatory mechanisms have been determined for several resistances (TABLE 2). Compensatory mutations can restore fitness by reducing the need for the affected function of the resistance protein, substituting the affected function with an alternative function or directly or indirectly restoring the efficiency of the affected function. For the compensation of resistance mutations, both substitution and restoration of function have been observed, and direct restoration of the function is by far the most common mechanism[28,29,39,92]. Below, we outline four interesting examples of compensatory mechanisms.

Compensation can occur by reversion of a mutation or by gene conversion. Although reversion to the wild-type genotype is rare, there are clinical examples of this occurring by gene conversion. In *S. aureus*, resistance to linezolid that was due to a mutation in a 23S ribosomal RNA gene (*rrl*) was lost in one case[93] and considerably reduced in another case[94] after removal of the antibiotic selective pressure. In both cases, resistance carried a fitness cost that could be reduced by gene conversion between the multiple copies of 23S *rrl* genes in bacteria with at least one wild-type copy. A similar mechanism explains heterogenous macrolide resistance in pneumococci[95].

Compensation can also occur by gene amplification. Gene duplications and amplifications can reversibly alter a resistance phenotype or its fitness. By creating a large genetic target, gene duplications and amplifications also increase the probability of mutations that can permanently alter the resistance phenotype and its fitness[9,10]. *Salmonella* spp. mutants that are resistant to actinonin, a peptide deformylase inhibitor, carry mutations in either of two genes that are required for the formylation of methionyl initiator tRNA (tRNAi): *folD* and the methionyl-tRNA formyltransferase gene (*fmt*). In the absence of antibiotic, the mutations reduced the fitness of bacteria grown in laboratory medium and in mice. The fitness costs could be ameliorated by intragenic mutations in *fmt* or *folD* or by extragenic compensatory mutations. One-third of the extragenically compensated *fmt* mutants carried amplifications of the identical, tandemly repeated *metZ* and *metW* genes, which encode the methionyl-tRNAi[96]. The increase in the copy number of *metZ* and *metW* (which was up to 40-fold higher than the copy number in bacteria without compensatory mutations) was accompanied by a similar increase in methionyl-tRNAi levels that compensated for the lack of Fmt activity and allowed translation initiation to proceed with non-formylated methionyl-tRNAi.

An increase in resistance can also be associated with compensation. Stepwise laboratory selection of increased fluoroquinolone resistance in *E. coli* showed that fitness decreased as up to five resistance mutations were accumulated. This correlation was occasionally broken, and an increase in resistance could be associated with an increase in fitness at a late selection step[58]. A similar reversal of the usual relationship was noted in strains of *Streptococcus pneumoniae* into which one or two

Gene conversion
A recombination event in which one strand of DNA is changed or repaired using information from another strand.

fluoroquinolone resistance mutations were introduced[97]. To establish cause and effect, a set of isogenic *E. coli* strains were constructed that carried up to five fluoroquinolone resistance mutations in different combinations[62]. Low-fitness triple mutants were identified in which the acquisition of a fourth resistance mutation substantially increased fitness *in vitro* and *in vivo* while simultaneously dramatically decreasing drug susceptibility. The largest effect occurred with the addition of a mutation in *parC* (which encodes DNA topoisomerase IV subunit A) to a low-fitness strain with resistance mutations in *gyrA* (which encodes DNA gyrase subunit A) and *marR* (which encodes multiple drug resistance protein R, a protein that regulates drug efflux) (FIG. 3b). Increased fitness was accompanied by a notable change in the level of activity of the *gyrA* promoter. Experiments performed in the absence of the antibiotic selected for *parC* mutants that improved fitness and reduced susceptibility to the antibiotic, implying that natural selection for improved growth in bacteria with low-level resistance to fluoroquinolones could also select for substantial reductions in drug susceptibility. Although further testing is required, a plausible mechanism is that *marR* mutations alter global transcription patterns and have negative effects on fitness, and the combination of mutations in *parC* and *gyrA* directly or indirectly restore transcription patterns, so restoring fitness, while also reducing fluoroquinolone binding to the DNA gyrase and topoisomerase, thereby increasing resistance to the drug. Thus, increased resistance to fluoroquinolones could be selected for even in the absence of further exposure to the drug[62]. Fitness compensation can also occur in clinical isolates, but does it occur in bacteria during growth in hosts and, if so, is such compensation important for stabilizing low-fitness, resistant bacterial strains? These questions are difficult to address experimentally, but compensation in bacteria isolated from patients has been suggested to occur in a few cases[25,39,68,98–101], indicating that compensatory evolution does occur outside the laboratory.

Physiological reasons for fitness costs. Understanding the physiological reasons for fitness costs may lead to an ability to predict these costs and to insights into potential weaknesses of resistant bacteria.

Two antibiotic resistance and fitness combinations in *S.* Typhimurium have been intensively studied both *in vitro* and in animal experiments; these are the Fus^R phenotype caused by mutations in EFG and Sm^R phenotype caused by mutations in the 30S ribosomal protein S12. These Fus^R mutants reduce the rate of protein synthesis but also have pleiotropic effects on bacterial physiology, because they perturb the accumulation of ppGpp in the cell[40]. As ppGpp is a global regulator of transcription, Fus^R mutants are defective in the induction of σ^S, have reduced virulence *in vivo*[41], have a reduced rate of respiration and are sensitive to oxidative stress[42]. Fus^R mutants are also hypersensitive to unrelated classes of antibiotics, including β-lactams, fluoroquinolones, aminoglycosides, rifampicin and chloramphenicol[43]. The costs of fluoroquinolone resistance in *E. coli* may also be closely associated with disturbed patterns of transcriptional regulation, possibly mediated through MarR and alterations in DNA supercoiling[62]. There is also a connection between fitness costs and transcriptional regulation in streptomycin resistance caused by mutant 30S ribosomal protein S12. Sm^R mutants have reduced rates of protein synthesis and reduced fitness *in vivo*[51,91], but some Sm^R mutants isolated in *S.* Typhimurium are also defective in σ^S induction, suggesting that the physiological effects of resistance are more complex and include altered global transcriptional regulation[31]. The detrimental effects of these fitness costs on the fates of resistant organisms in natural populations could potentially be exploited for designing drugs, therapeutic regimes and intervention strategies.

The high fitness cost associated with induction of VanA-type resistance in *S. aureus*[57] suggests that this feature could be exploitable in the design of therapies. For example, the effectiveness of treatment might be increased by combining chemical induction of *vanA* overexpression with the use of an antibiotic to which the bacteria are susceptible. It is not known how the cost of *vanA* induction is manifested, although the obvious possibilities include the costs of expressing the *vanA* operon proteins. Alternatively, the additional costs associated with transglycosylation, transpeptidation or transport of D-alanyl–D-lactate rather than D-alanyl–D-alanine. The clinical importance of vancomycin argues that understanding the physiological basis of fitness costs in isolates with VanA-type resistance should be a priority.

Real-life approaches to test reversibility

The complexity of bacterial population dynamics means that we require clinical studies to address the question of whether reversibility is feasible in reality. Studies have been performed to test the reversal of resistance in individuals, in community settings and in hospitals. At the levels of the individual and community, the biological cost is thought to be the main driving force for the reduction in the frequency of resistant bacteria in the absence of antibiotics[7,23,89]. By contrast, in hospital settings the driving force is proposed to be a dilution effect caused by the continuous influx of patients who are either uninfected or infected with susceptible bacteria. In hospitals, both modelling[7,23,102–104] and analysis of the correlations between antibiotic resistance and variation in antibiotic use[105–107] show that alterations in antibiotic use can cause rapid changes (in the order of days to months) in the frequency of resistance. By contrast, when the fitness cost of resistance is the main driving force behind its reversal, the rate of change is expected to be much slower (months to years). Thus, after removal of the selective pressure in community settings, even without complicating factors such as compensatory evolution and co-selection, a long time may pass before a reduction in the frequency of resistance is seen[7,23,89]. Below, we discuss experiments and intervention studies that attempted to reverse resistance. For reversibility studies carried out in individuals, the principle is to follow the frequency of resistant bacteria in an individual before and after an antibiotic treatment that selects for an increased frequency of resistant bacteria and to

determine how rapidly that frequency is reduced after the antibiotic pressure is relieved. The rationale is that, over time, susceptible bacteria (either survivors of the treatment or imported from the environment), which are more fit, will outcompete the selected resistant bacteria, which are less fit, when antibiotic pressure is absent. In two studies performed on five dyspeptic individuals who had been treated for 1 week with the macrolide clarithromycin for *Helicobacter pylori* infection, the selection and reversion of clarithromycin-resistant (Cla^R) enterococci in the intestine and Cla^R *Staphylococcus epidermidis* on the skin was followed[108,109]. As expected, in these five treated patients, all enterococci that were isolated before treatment were susceptible to the drug, but those recovered immediately after treatment showed high-level resistance, which was due to the introduction of *ermB* (encoding erythromycin resistance protein B). In three of the patients, the selected resistant enterococci persisted for 1 to 3 years without any further antibiotic selection (that is, the patients received neither clarithromycin nor any other antibiotic after the initial clarithromycin treatment). Similarly, for *S. epidermidis* all strains isolated immediately after treatment were clarithromycin-resistant (owing to the introduction of *ermC*), and the same resistant strains persisted for up to 4 years in three patients in the absence of further antimicrobial treatment. These studies show how antibiotic treatment selects for resistance in the indigenous microflora and that these resistant bacteria can persist for years without any further antibiotic selection. By contrast, the overall species composition of the microflora can be quite rapidly restored after ecological disturbances that are induced by treatment with certain classes of antibiotic, such as pivmecillinam[110]. The rate of reappearance of susceptible strains after removal of the antibiotic pressure will depend on several factors (including relative fitness, the turnover rate of bacteria and the supply rate of susceptible strains), and it is therefore likely that reversibility rates will vary depending on the individual, the bacterial species and the antibiotic resistance mechanism. At the community level, three nationwide interventions have been performed in which the effects of reduced antibiotic use on resistance were analysed retrospectively. A reduction in the use of certain macrolide antibiotics in Finland was followed by a substantial decrease in erythromycin-resistant *Streptococcus pyogenes*[111]. Similarly, in a study performed in Iceland the decreased use of antibiotics in children was followed within a few years by a decrease in penicillin-resistant *S. pneumoniae*[112]. However, subsequent analysis suggested that for both studies the reduction in resistant bacteria might have been caused by clonal replacements that were unrelated to the reduction in antibiotic use, illustrating the importance of knowing the clonal structure of the bacterial population both before and after the intervention[113,114]. Furthermore, for the erythromycin resistance study, no analysis was carried out to determine the magnitude of the fitness costs, although such data would be required to explain the rapid alterations in the frequency of resistance and how they related to antibiotic use. A modelling analysis using the penicillin resistance study suggested that resistance did not reduce bacterial fitness in the host but notably increased the ability of the resistant bacteria to be transmitted between hosts relative to the susceptible strains[115]. To our knowledge, these conclusions have not been further examined experimentally. In contrast to the dramatic effects seen in these two studies, a 97% decrease in consumption of co-trimoxazole in the United Kingdom between 1991 and 1999 did not result in a reduction in sulfamethoxazole resistance[116]. A follow-up study in 2004 based on additional data from the same area that also included data on streptomycin resistance showed that sulfamethoxazole and streptomycin resistance in *E. coli* had remained stable despite a continued low-level use of these drugs[117]. The lack of effect on resistance was attributed to a low or non-existent fitness cost of resistance and to co-selection, such that sulfamethoxazole and streptomycin resistances are maintained by their close genetic linkage to another selected antimicrobial resistance.

To control for the undefined aspects of these three studies (for example, a poor description of the bacterial clonal structure and the pattern of antibiotic use, as well as the lack of a control group and of a long-term baseline for the intervention), a large prospective intervention study was performed. A 24-month voluntary restriction was introduced on the use of trimethoprim-containing drugs in Kronoberg County, Sweden, with a baseline of 14 years of monthly data on trimethoprim resistance and consumption to determine if the frequency of trimethoprim-resistant *E. coli* from urinary tract infections would be reduced[118]. For large subsets of collected *E. coli*, the fitness cost of trimethoprim resistance, the presence of any associated resistances and the clonal composition of *E. coli* before and after the intervention were determined. As compared with a neighbouring control county, there was a prompt and sustained 85% reduction in the use of trimethoprim-containing drugs during the intervention, and this reduction was associated with a marginal but statistically significant effect on trimethoprim resistance. No changes were observed in the clonal composition of *E. coli* before, during or after the intervention, and there was no measurable fitness cost associated with trimethoprim resistance, as determined by *in vitro* measurements of growth rates. The frequency of associated antibiotic resistances in trimethoprim-resistant isolates was high, and most bacteria were resistant to one or more of the other antibiotics used for treatment of urinary tract infections. In conclusion, a drastic 2-year reduction in trimethoprim use had a very small effect on the frequency of trimethoprim resistance in *E. coli*[118]. This can be explained by the low fitness cost of trimethoprim resistance combined with the co-selection that was due to high levels of associated resistance, but at present the relative contributions of these two effects cannot be assessed. Whether this conclusion can be generalized to other resistances and bacteria is debatable, but unless the period of non-use is much longer than 2 years, the cost of resistance is much higher than that for trimethoprim resistance in *E. coli*, or the potential for co-selection is low, then

Baseline
Value for the frequency of resistance before the intervention.

we would predict that it is unlikely that any clinically significant reduction in resistance will be observed for similar attempts.

Conclusions and perspectives

Studies of fitness costs and the dynamics of resistance development in various pathogenic bacteria have led to several conclusions of medical and biological interest. The main finding is that reversibility in clinical settings is expected to be slow or non-existent. The main reasons for this are that the intrinsic dynamics of reversal are expected to be slow (even if a fitness cost is present), compensatory evolution and cost-free resistances can reduce the cost and thereby reduce the driving force for reversibility, and co-selection between the resistance mechanism and other selected markers can slow down any potential reversibility that may be driven by fitness costs. A second key conclusion is that we could use this knowledge to reduce the likelihood of resistance development, for example, by choosing antibiotic targets for which the resistance mechanism confers a high fitness cost and for which the rate and extent of compensation is low. Similarly, it could be possible to exploit the detailed knowledge of the physiological basis of fitness costs for the choice and design of novel therapies that target the physiological 'Achilles heel' that is associated with a particular resistance mechanism. Finally, a better understanding of fitness costs and compensatory evolution and of their impact on the emergence and spread of resistant bacteria should allow us to make better quantitative predictions about the rate and trajectory of the evolution of resistance towards new and old drugs and, by inference, should also give us possibilities to prevent this evolution.

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Competing interests statement
The authors declare no competing financial interests.

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