REVIEW ARTICLE

# Persistence of antibiotic resistance in bacterial populations

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## Abstract

Unfortunately for mankind, it is very likely that the antibiotic resistance problem we have generated during the last 60 years due to the extensive use and misuse of antibiotics is here to stay for the foreseeable future. This view is based on theoretical arguments, mathematical modeling, experiments and clinical interventions, suggesting that even if we could reduce antibiotic use, resistant clones would remain persistent and only slowly (if at all) be outcompeted by their susceptible relatives. In this review, we discuss the multitude of mechanisms and processes that are involved in causing the persistence of chromosomal and plasmid-borne resistance determinants and how we might use them to our advantage to increase the likelihood of reversing the problem. Of particular interest is the recent demonstration that a very low antibiotic concentration can be enriching for resistant bacteria and the implication that antibiotic release into the environment could contribute to the selection for resistance.

## Introduction

### Scope of the review

Antibiotic concentrations in the environment are the main driving force for the selection of resistant bacterial populations. Antibiotic resistance mechanisms are usually associated with fitness costs for bacteria and should reduce the frequency of resistance in the absence of antibiotic selection, but compensatory evolution can potentially restore fitness without loss of acquired resistance. Thus, the continued maintenance of antibiotic resistance in a bacterial population is expected to be a function of the rates and magnitudes of competing forces selecting for and against resistance. In addition, there is a possibility that some resistance mechanisms might be cost-free. This review will consider and evaluate examples for which there is experimental evidence relevant to the reversibility and persistence of antibiotic resistance in bacterial populations. We will consider the roles played by compensatory evolution, cost-free mutations, genetic coselection and plasmid-specific mechanisms in slowing the reversibility of antibiotic resistance.

### Framing the problem of antibiotic resistance

The introduction of antibiotics in the mid-20th century was arguably the single most important medical event in recent history with regard to reducing human morbidity and mortality. However, the subsequent and continuing intensive use of antibiotics, both in medicine and in agriculture, estimated to total several million tons worldwide since their introduction (Wise, 2002; Andersson & Hughes, 2010), has helped to select a huge increase in the frequency of resistance among human pathogens. High frequencies of resistance significantly reduce the possibility of effectively treating infections. This increases the risk of complications and fatal outcome (Helms *et al.*, 2002; Depuydt *et al.*, 2008), increases the economic burden on health care systems (Cosgrove, 2006; Sipahi, 2008; Roberts *et al.*, 2009) and may ultimately threaten a postantibiotic era (Guay, 2008; Lew *et al.*, 2008; Woodford & Livermore, 2009).

### Questions of persistence and reversibility

A rational approach to dealing with antibiotic resistance problems requires detailed knowledge of the different biological and nonbiological factors that affect the rate and extent of resistance development. On the one hand, this will require quantitative information on antibiotic selective pressures and on bacterial population structures and how the interplay between these influences rates of resistance development. On the other, it will also be necessary to

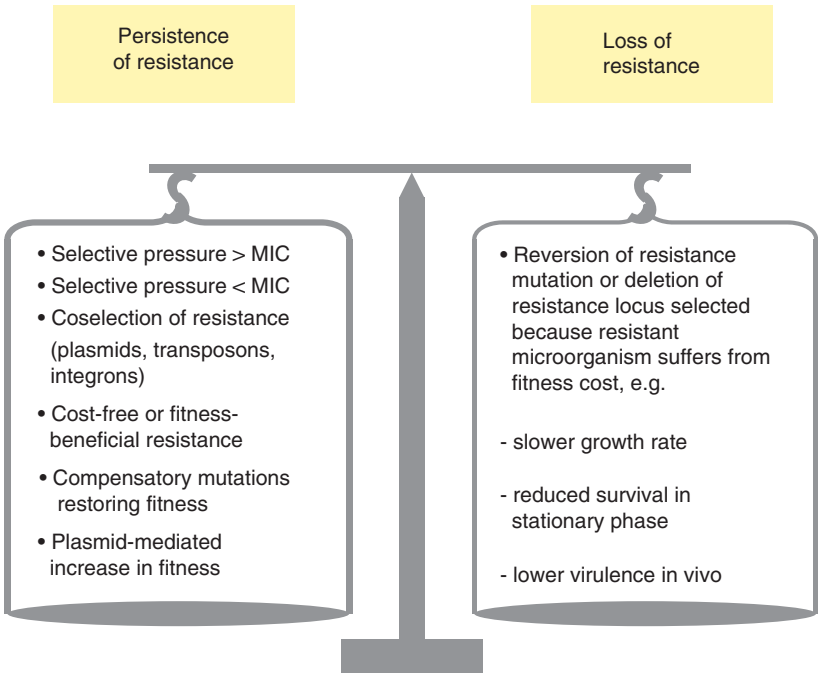


**Fig. 1.** Various selective forces and mechanisms that will act to either cause loss or persistence of resistance.

quantify the influence of resistance on bacterial fitness and to determine the relationships between fitness costs and changes in the frequency of resistant pathogens. It is noteworthy that acquired antibiotic-resistance traits are frequently found in bacteria isolated from humans and animals that are not known to have been subjected to significant antibiotic exposure and that live in remote parts of the world (Pallecchi *et al.*, 2008). In this context, one of the most important issues is to understand how resistance is stabilized in bacterial populations, in particular under environmental conditions where antibiotic selective pressure may be very low or absent for significant periods of time. In theory, resistance might be stabilized by (1) compensatory mutations that restore fitness without loss of resistance, (2) by the occurrence of rare cost-free resistance mutations or (3) by genetic linkage and coselection between resistance mutations and other selected genetic markers (e.g. virulence factors or resistances) as well as by other factors (Fig. 1). There is experimental evidence that supports the relevance of each of these stabilization mechanisms and we will discuss some examples in later sections. There are also a number of theoretical studies modeling the relationship between the biological cost of resistance, the rate by which resistance develops and the steady-state frequency of resistance at a given antibiotic pressure as well as the rate by which resistance is predicted to disappear if antibiotic use is reduced (Levin *et al.*, 1997; Andersson & Levin, 1999; Austin & Anderson, 1999; Austin *et al.*, 1999; Levin *et al.*, 2000; Levin, 2001, 2002; Andersson, 2003). In addition, there are factors that will confound these models (e.g. clonal shifts and changes in population structure unrelated to antibiotic selection) affecting the expected relationships and making predictions less certain (Andersson & Levin, 1999; Austin & Anderson, 1999; Austin *et al.*, 1999; Levin *et al.*, 2000; Levin, 2001; Andersson, 2003). Finally, we will describe the outcomes of laboratory studies and clinical interventions to reduce the frequency of resistant bacteria at the community level and explain why reversibility, if it occurs at all, will occur so slowly that it in most cases, it is unlikely to be of practical importance.

## Driving forces for reversibility

When a resistance problem has been generated and the frequency of resistance has reached a level that prohibits the successful use of empirical therapy, any potential for reversibility of the problem will be determined by essentially two factors – dilution and the fitness cost – depending on whether one considers open or closed systems. Examples of an open system would be a hospital or an animal farm where there is a continuous in- and out-flow of humans or animals and where the incoming population has a lower frequency of resistance than the resident population (which is generally exposed to a higher antibiotic pressure). If antibiotic use is reduced in the hospital/animal farm, the driving force for reversibility is essentially a dilution effect where the incoming population reduces the frequency of resistance in the hospital/farm. This notion is supported by mathematical modeling (Austin & Anderson, 1999; Lipsitch *et al.*, 2000; Bonten *et al.*, 2001) as well as by clinical interventions where

quite rapid (weeks to months) reductions in resistance can be observed after the use of specific antibiotic has been decreased (McGowan, 1986; Mahamat *et al.*, 2007; Aldeyab *et al.*, 2008). In a closed system, for example in a community where migration is limited, the main factor determining whether antibiotic-resistant populations can be displaced with antibiotic-susceptible ones is the biological fitness cost of resistance, i.e. any effect the resistance mechanism has on reducing the ability of the pathogen to reproduce and spread in the host population. Both theoretical arguments (Levin *et al.*, 1997, 2000; Levin, 2001; De Gelder *et al.*, 2004) and clinical data (Bottger *et al.*, 1998; Sander *et al.*, 2002; Cohen *et al.*, 2003) support the notion that the larger the fitness cost, the lower the frequency of resistant bacteria in the population at a given antibiotic pressure. Similarly, if antibiotic use is reduced, the resistant mutants with the highest fitness costs are expected to be outcompeted and disappear the fastest, but unfortunately, if no fitness cost is present, the resistant mutants will remain even if antibiotic use is discontinued.

## Factors that will cause persistence

*1. Direct selection for resistance marker in the presence of high drug concentrations [above the minimal inhibitory concentration (MIC)].* The rate of appearance of antibiotic-resistant bacteria in a susceptible population will be a function of the combined rates of *de novo* mutation, which need not be high (Örlén & Hughes, 2006), and of the horizontal gene transfer (HGT) of resistance determinants. However, the more important measure is the rate at which existing resistant variants increase in frequency as a function of the level of drug exposure in pathogen populations (Bergman *et al.*, 2004, 2009). The level of exposure in clinical settings will be associated with pharmacokinetic and pharmacodynamic aspects of the drug in patients that affect clearance of the pathogen or selection of resistant variants within the patient (Olofsson *et al.*, 2007), and also with hygienic and transmission control measures in clinics and hospitals. In addition, the volume and distribution of antibiotics released into the wider physical environment can be expected to contribute to the selection of resistance and transfer of resistance by HGT. This latter point is important because the large reservoir of resistance genes, even within the human microbial community, could potentially serve as donors for transfer of resistance to human pathogens (Sommer *et al.*, 2009).

*2. Sub-MIC selection.* While it is evident that antibiotic concentrations above the MIC of the bacteria can select for resistant variants, it still largely remains unclear whether levels far below MIC can be selective. Because many natural environments and body compartments will be exposed to antibiotic concentrations $\ll$ MIC, due to anthropogenic pollution (Thiele-Bruhn, 2003; Cabello, 2006; Li *et al.*, 2008; Kummerer, 2009) or antibiotic treatments (Drlica, 2003; Drlica & Zhao, 2007), there is in theory also ample opportunity for resistance to be selected under sub-MIC conditions (Baquero *et al.*, 1997; Negri *et al.*, 2000) because of very small reductions in the growth rate of the susceptible strains (Fig. 2). Recent results suggest that this process might be more important than previously realized. Thus, we have observed for several well-defined, clinically common mutants of *Escherichia coli* and *Salmonella enterica* with resistance to three different classes of clinically used antibiotics (tetracyclines, fluoroquinolones and aminoglycosides) that selection of resistant bacteria can occur at antibiotic concentrations up to several hundred-fold below the MIC of the susceptible bacteria, corresponding to absolute antibiotic concentrations in the $\mu g\,mL^{-1}$ to $pg\,mL^{-1}$ range (E. Gullberg *et al.*, unpublished data). These results were obtained using competition experiments between pairs of susceptible and resistant strains that were genetically tagged with green fluorescent protein (GFP) and counted by fluorescence-activated cell sorting. This experimental setup allowed the detection of growth rate differences as small as 0.3% (Lind *et al.*, 2010). Furthermore, using a different experimental setup, recent results from Liu *et al.* (2011) report similar findings. In conclusion, these results imply that the ultralow ($\ll$ MIC) antibiotic concentrations found in many natural environments are, in contrast to common belief, sufficiently high to confer the selection and persistence of antibiotic resistance.

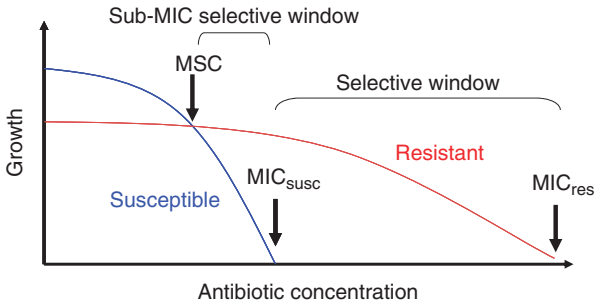


**Fig. 2.** Schematic representation of growth rates as a function of antibiotic concentration. The sub-MIC and traditional selective windows where the resistant strain will outcompete the susceptible strain are indicated. MICsusc, minimal inhibitory concentration of the susceptible strain; MICres, minimal inhibitory concentration of the resistant strain; and MSC, minimal selective concentration where the resistant mutant is enriched over the susceptible strain.

*3. Coselection between resistance markers (within genetic elements and within clones).* A resistance marker could increase in frequency by virtue of its genetic linkage to a selected marker in the population and coselection is a common feature of resistance that is acquired by HGT. This is clearly of importance with regard to plasmids, transposons and integrons, but it also applies to the carriage of resistance in the context of any multiresistant clone. Because

of genetic linkage, the frequency of resistance to a particular antibiotic could in theory remain stable or even increase in environments where the antibiotic might not currently be in use. The causes governing the dynamics of an unselected resistance marker in a bacterial population could be many. Selection might be for another resistance determinant or for a determinant of clonal fitness not directly related to antibiotic resistance. Regardless of the particular cause, the implication of coselection is that those strains that increase in the population do so because they are more fit in the presence of the antibiotics that are in use. There are examples in the literature where there is little or no correlation between changes in the consumption of a particular antibiotic and the observed changes in the frequency of resistance and this has in some cases been tentatively attributed to coselection. For example, a recent intervention study in Kronoberg County in Sweden, where the use of trimethoprim-containing drugs was decreased by 85% during the 24-month period of the study, reported only a marginal effect on trimethoprim-resistance levels in *E. coli* (Sundqvist *et al.*, 2009). The authors calculated that it would take a sustained intervention for 12 years just to bring the level of resistance from the 12% measured at the start of the intervention back to the level of approximately 8% measured in 1991. The reasons for this disappointingly small effect were reasoned to be due to a combination of the small fitness cost measured for trimethoprim-resistance together with a strong coselection for other antibiotics (e.g. mecillinam, furantoin, fluoroquinolones, cephalosporins, etc.), which remained in use during the intervention period. Similarly disappointing results were also recorded in an earlier study from Great Britain when the use of sulfonamides was reduced. The consumption of cotrimoxazole in the United Kingdom was reduced by 97% between 1991 and 1999, but this did not result in a reduction in sulfamethoxazole resistance (Enne *et al.*, 2001). A follow-up study based on additional data from the same area in 2004 showed that sulfamethoxazole and streptomycin resistance in *E. coli* had remained stable despite a continued low use of these drugs (Bean *et al.*, 2005). The absence of an effect on resistance was attributed in part to the surprising observation that at least one of the clinical plasmids carrying sulfonamide resistance actually increased fitness for *E. coli* in laboratory tests (Enne *et al.*, 2004). An additional contributing factor to the observed coselection was that sulfamethoxazole resistance was genetically linked to other resistance genes that continued to be selected during the study period. In two earlier nationwide studies carried out in Finland and Iceland, the effects of reduced antibiotic use on resistance were analyzed retrospectively (Kristinsson, 1997; Seppälä *et al.*, 1997). In both studies, decreases in antimicrobial resistance frequencies were noted. A reduction in the use of macrolides in Finland was followed by a significant decrease in erythromycin-resistant *Streptococcus pyogenes* while in Iceland a decrease in *Streptococcus pneumoniae* resistant to penicillin followed a decrease in the use of antibiotics in children a couple of years earlier. However, later reanalysis of the data suggested that for both studies, the reduction might have been caused by clonal replacements unrelated to the reduction in antibiotic use (Kataja *et al.*, 1999; Arason *et al.*, 2002). The actual driving force for this clonal replacement is not known, but logic suggests that it is likely to be a consequence of the different fitness profiles of the different clones involved; however, this has not been tested.

*4. Cost-free resistances.* To determine whether a resistance mechanism is truly cost-free for a bacterium is difficult because it can always be argued, in spite of repeated demonstrations that a resistance appears cost-free under the tested conditions, that there is some environmental condition where a cost might be present. In addition, limitations of the experimental setups will also make claims of cost-free resistances difficult to confirm. By single culture/animal growth experiments, differences usually have to be $> 5\%$/several-fold to be reliably detected. The resolution can be increased considerably by performing competition experiments, and by utilizing GFP tagged and pre-adapted strains, differences as small as 0.3% can be identified (Lind *et al.*, 2010). However, differences smaller than 0.3% are extremely difficult to detect due to the phenomenon of periodic selection, where one of the competitors acquires an adaptive mutation during the competition, resulting in a noninterpretable experiment. Thus, claims that have been made that certain mutations appear to be cost-free need to be evaluated with the above limitations and caveats in mind (Ramadhan & Hegedus, 2005; Criswell *et al.*, 2006). One example of a mutation that has been suggested to be cost-free, based on *in vitro* and animal experiments, is streptomycin resistance caused by the substitution K42R in ribosomal protein S12 (Tubulekas & Hughes, 1993; Kurland *et al.*, 1996; Bjorkman *et al.*, 1998; Enne *et al.*, 2005). This substitution is also found at a high frequency in streptomycin-resistant clinical isolates of *Mycobacterium tuberculosis* (Bottger *et al.*, 1998; Sander *et al.*, 2002). However, highly sensitive competition experiments (see Driving forces for reversibility) show that this mutation does confer a small cost (about 3%) that has gone undetected previously. Whether this cost is medically relevant with regard to the short/long-term persistence of the mutation in a pathogen population is unclear, but theoretically, one would expect a selection coefficient of $-0.03$ for a mutation to provide a significant counter-selective force in most bacterial populations.

*5. Fitness-increasing resistances.* Although it is normally observed that antibiotic resistance is associated with a fitness cost for bacteria in the absence of drug selection, it is in principle possible that resistance could increase fitness in drug-free environments. An example of this type has

recently been demonstrated with regard to resistance to fluoroquinolones in *E. coli* (Marcusson *et al*., 2009). Resistance to fluoroquinolones develops in *E. coli* in a multistep process, typically including the acquisition of efflux mutations and several different mutations altering drug-target proteins (Heisig, 1996; Komp Lindgren *et al*., 2003, 2005). In addition, there is increasing evidence that a large variety of fluoroquinolone-resistance genes and mechanisms can be acquired by HGT events (Martinez-Martinez *et al*., 1998; Robicsek *et al*., 2006; Perichon *et al*., 2007; Jacoby *et al*., 2008). Each individual resistance mutation, gene or mechanism contributes only to a very small increase in MIC and clinically relevant levels of resistance always require the accumulation of several alterations. In step-wise laboratory selections, the accumulation of up to five fluoroquinolone resistance mutations in *E. coli* was shown to be generally associated with significantly reduced fitness (Komp Lindgren *et al*., 2005). However, in a few of the evolved lineages, this monotonous relationship was broken and an increase in resistance was found to be associated with an increase in fitness at a late selection step (Komp Lindgren *et al*., 2005). A reversal of the expected fitness–resistance relationship was also noted in constructed strains of *S. pneumoniae* carrying one or two fluoroquinolone resistance mutations (Rozen *et al*., 2007). To establish the relationship between cause and effect, a set of isogenic *E. coli* strains were constructed carrying, in different combinations, up to five fluoroquinolone-resistance mutations (Marcusson *et al*., 2009). Within this set of constructed isogenic strains, low-fitness triple-mutants were identified, where the addition of a fourth resistance mutation significantly increased fitness *in vitro* and *in vivo* while at the same time dramatically decreasing drug susceptibility (Marcusson *et al*., 2009). The largest effect was observed when a *parC* mutation (topoisomerase IV) was added to a low-fitness strain carrying resistance mutations in *gyrA* (DNA gyrase) and *marR* (upregulating drug efflux). Increased fitness was accompanied by a significant change in the level of *gyrA* promoter activity. When the same low-fitness triple mutants were subjected to selection and competition experiments performed in the absence of drug, spontaneous *parC* mutations that improved fitness and reduced drug susceptibility were also selected. The implication of these data is that natural selection for improved growth in low-fitness bacteria with a low-level resistance to fluoroquinolones can select for reduced drug susceptibility in the absence of drug selection (Marcusson *et al*., 2009).

*6. Compensatory evolution reduces costs and allows maintenance even without selective pressure.* The fitness costs of drug resistance can be reversed, at least partially, by compensatory mutations without any significant loss of resistance (Andersson & Hughes, 2010). Thus, several *in vitro* and *in vivo* studies show that when resistant bacteria are serially passaged, compensatory mutations are easily selected, although the degree of restoration varies and is usually below that of the original susceptible strain. However, in principle, by the reiteration of cycles of selective compensation, resistant clones could become as fit as susceptible clones. Accordingly, genetic compensation could act to stabilize and maintain resistant populations even in the absence of antibiotic selection. The details of which mutations will be associated with genetic compensation for any particular drug–bacteria combination should depend on the interplay of mutation rates, fitness of different mutants and bottleneck size during serial transfer (Levin *et al*., 2000; Maisnier-Patin *et al*., 2002; Handel *et al*., 2006). This general expectation is supported by experimental data from studies of fusidic acid-resistant *fusA* mutants and streptomycin-resistant *rpsL* mutants in *S. enterica* ser. Typhimurium (Johanson *et al*., 1996; Bjorkman *et al*., 1998, 1999, 2000; Nagaev *et al*., 2001; Maisnier-Patin *et al*., 2002). Thus, genetic compensation occurs much more frequently than reversion. This bias in favor of new mutations is also expected to be relevant for natural populations, when population bottlenecks are present. Although in principle new mutations could restore fitness by a variety of different mechanisms, direct restoration of the impaired function is, according to the available data, the most common mechanism (Johanson *et al*., 1996; Bjorkman *et al*., 1998, 2000; Nagaev *et al*., 2001).

Here, we outline four illustrative examples of different fitness compensatory mechanisms. The first example concerns a case of apparent reversion to the wild-type genotype that actually occurs by a process of gene conversion. In *Staphylococcus aureus*, resistance to linezolid caused by a mutation altering a 23S rRNA gene was observed in one case to be lost (Meka *et al*., 2004b) and in the other case to be considerably reduced (Meka *et al*., 2004a), after the removal of the antibiotic selective pressure. In both cases, linezolid-resistance carried a fitness cost and the loss or the reduction in the resistance phenotype was caused by gene conversion between the multiple copies of 23S *rrn* genes where at least one copy had remained wild type in sequence. In the above examples, selection for increased fitness is leading to reversion of resistance. However, gene conversion works both ways and in an environment where selection favored increased resistance, for example in the presence of low levels of antibiotic, gene conversion would provide a rapid genetic and phenotypic solution, providing at least one gene copy carried a pre-existing resistant allele. A similar gene conversion mechanism could explain the phenomenon of heterogenous macrolide resistance observed in pneumococci (Wolter *et al*., 2006). Although it is not currently in clinical use, resistance to kirromycin, an antibiotic that targets the translation elongation factor EF-Tu, is also strongly influenced by gene conversion because EF-Tu is produced in

many bacteria from duplicate *tufA* and *tufB* genes (Hughes, 1990). Depending on the type of selection pressure that is imposed, either for increased drug resistance, or for increased fitness, the process of *tuf* gene conversion can either copy a resistance mutation to the second *tuf* gene or it can restore a wild-type sequence to both *tuf* genes (Abdulkarim & Hughes, 1996; Hughes, 2000). This makes the development of resistance to drugs that target the products of repetitive genes (such as rRNAs and EF-Tu) highly unstable. On the one hand, resistance is very unlikely to arise in one step because it is recessive and would require multiple mutations, but on the other, the effects of a single small effect mutation may be rapidly amplified by gene conversion.

In the second example, environment and epistasis have been shown to play significant roles in determining the magnitude of fitness costs associated with antibiotic resistance. The fitness costs of streptomycin resistance ($Sm^R$) caused by mutations in *rpsL* (encoding ribosomal protein S12) is partly caused by reduced rates of protein synthesis affecting growth rates *in vivo* and *in vitro* (Bjorkman *et al.*, 1999), but it can also depend on environmental and epistatic effects (Paulander *et al.*, 2009). Thus, the *Salmonella typhimurium* $Sm^R$ mutants K42N and P90S have impaired growth on a rich medium, but in media with poorer carbon sources, the same $Sm^R$ mutants grow faster than the wild type. The reason appears to be that the drug-resistant mutants do not induce the stress-inducible sigma factor RpoS ($\sigma^S$), a key regulator of many stationary phase- and stress-inducible genes, and thus escape the growth inhibition that this induction imposes on drug-susceptible wild-type cells under conditions of environmental stress and starvation (Paulander *et al.*, 2009). In another example, a mutation in *gyrA* that reduces susceptibility to ciprofloxacin in *Campylobacter jejuni* enhances the fitness of the mutant strain in competition against an isogenic drug-susceptible strain in a chicken infection model, in the absence of drug selection (Luo *et al.*, 2005). However, the same mutation in a different strain of *C. jejuni* imposes a fitness cost. These examples show that fitness costs can be influenced by the environment in which they are measured and raise the hope that by imposing suitable environmental conditions, it might be possible to increase the selection against some drug-resistant pathogens. The influence of epistatis in modulating the magnitude of fitness costs may be a relatively common feature of drug resistance that is currently under-appreciated simply because of a lack of relevant research.

Modulating the potential costs of resistance by the appropriate regulation of gene expression may be of critical importance in maintaining resistance genes in the absence of direct drug selection. Drug resistance plasmids usually carry a fitness cost in terms of a reduced growth rate, at least at the stage when they enter naïve bacteria (Lenski & Bouma, 1987; Bouma & Lenski, 1988; Smith & Bidochka, 1998; Johnsen *et al.*, 2002; Dahlberg & Chao, 2003). The nature of these costs may vary and, in most cases, has not been determined, but it is likely that the costs are strongly associated with the disruptive physiological effects of introducing multiple new biochemical pathways into the host bacteria. Compensatory evolution following plasmid acquisition by naïve *E. coli* has been shown experimentally to occur both on the plasmid and on the chromosome and to create genetic combinations for which fitness would be reduced if the plasmid were subsequently lost (Dahlberg & Chao, 2003). In such situations, the plasmid carrying drug-resistant genes may be maintained stably in the bacteria even in the absence of selection with antibiotics. An example relevant to both epistasis and the importance of gene regulation concerns the AmpC-type β-lactamase. The gene for *ampC* is not intrinsic to the chromosome of *Salmonella* spp. and the AmpC-resistance phenotype was rarely found in clinical isolates. When *ampC* was experimentally transformed on a plasmid into *Salmonella*, it reduced the growth rate and invasiveness, suggesting reduced fitness as a plausible reason for its rarity (Morosini *et al.*, 2000). However, when both *ampC* and its regulator *ampR* were introduced (making β-lactam resistance inducible rather than constitutive), these fitness costs were eliminated (Morosini *et al.*, 2000). In this example, the genetic context of the resistance gene, and the physiological costs of expressing the gene, determined the relative fitness cost associated with its acquisition. However, this fitness barrier has not prevented the spread of *ampC*-β-lactamases to *Salmonella*. Plasmid-borne AmpC-type enzymes are now found widely distributed in veterinary and clinical isolates; reviewed in (Jacoby, 2009). Interestingly, not all plasmids bearing *ampC* carry the regulator gene *ampR* and experimental measurements suggest that the biological cost of high-level AmpC production can be compensated by other plasmid-encoded functions (Hossain *et al.*, 2004). Another example in which appropriate gene regulation has been shown to reduce the cost of resistance is illustrated by VanA-type glycopeptide resistance in *S. aureus* (Arthur *et al.*, 1996). Resistance is acquired by HGT of a *vanA* resistance operon and results from the synthesis of alternative cell wall precursors (ending in D-Ala-D-Lac) with a low affinity for glycopeptides and the elimination of the normal susceptible precursors (ending in D-Ala-D-Ala) to which vancomycin binds (Bugg *et al.*, 1991). Resistance is induced in response to glycopeptides (Arthur *et al.*, 1992). When it was shown that the *vanA* gene had crossed the genus boundary from vancomycin-resistant enterococci to methicillin-resistant *S. aureus* (MRSA), it was feared that it would spread rapidly, representing an immediate threat to patient care and with the possibility of spreading to other, more virulent pathogens (Willems *et al.*, 2005). However, VanA-positive *S. aureus* have not spread in the clinical

setting as initially feared and the number of MRSA strains that are also resistant to vancomycin remains very small (Perichon & Courvalin, 2009). Pairwise comparisons of isogenic MRSA strains with and without three different clinical isolates of the VanA resistance operon showed that when induced, the fitness cost was large and reduced the growth rates by 20–38% (Foucault *et al*., 2009). In contrast, in the absence of induction, the fitness cost of carrying the VanA operon was only 0.04–0.3%. Thus, VanA-type resistance can persist because it has a low cost in the absence of the drug and although it is costly in absolute terms when induced by the drug, it is also essential for bacterial survival under those conditions. This example clearly illustrates the importance of gene regulation and physiology in considering the potential and real costs of antibiotic resistance for bacteria. It is likely that the costs initially associated with many plasmids and other HTG sequences are related to the disruption they cause to growth physiology by inappropriate patterns of gene expression and that to survive in the absence of drug selection, they must adapt to the host bacteria by restoring an appropriate physiological balance to their gene expression.

Finally, the genetic flexibility associated with genetic duplication and amplification (GDA) events may be important because it increases the probability of certain resistances arising and also because it can serve as a crude way of regulating the costs of resistance in the absence of selection. By increasing genetic target size GDA increases the probability of occurrence of mutations that can permanently alter the resistance and/or fitness phenotypes (Andersson & Hughes, 2009; Sandegren & Andersson, 2009). GDA can also reversibly alter resistance and fitness phenotypes. For example, *Salmonella* mutants resistant to the peptide deformylase inhibitor actinonin carry mutations in either of two genes required for the formylation of methionyl initiator tRNA (tRNAi): *fmt* and *folD*. In the absence of antibiotic selection, these mutations reduce fitness. It has been shown experimentally that approximately one-third of the extragenically compensated *fmt* mutants carried amplifications of the tandemly repeated *metZ* and *metW* genes, encoding the initiator tRNA (Nilsson *et al*., 2006). The increase in *metZ* and *metW* gene copy number was by up to 40-fold, increasing tRNAi levels and compensating for the lack of methionyl-tRNA formyltransferase activity. The advantages of GDA mechanisms are that they are very frequent, they facilitate the selection of relevant mutations by increasing target size and they are reversible in situations where selection pressure is subsequently relaxed.

*7. Plasmid persistence*. Plasmids typically carry genes that are conditionally beneficial and as a result plasmids are expendable under many conditions. Therefore, the continued maintenance of plasmids requires either a continuous selection (e.g. for antibiotic or heavy metal resistance genes) or the presence of other mechanisms that ensure persistence. Apart from the previously mentioned processes (1–6), which can confer persistence to both chromosome- and plasmid-encoded resistance mechanisms, there exist several plasmid-specific mechanisms that ensure plasmid maintenance even in the absence of direct selection. For example, several interesting cases have been described where plasmids confer increased fitness in the form of faster growth rates in the absence of any selective pressures. Thus, both conjugative and nonconjugative plasmids (carrying various antibiotic resistance genes) can substantially increase bacterial exponential growth rates, implying that the plasmids are maintainable without drug selection (Enne *et al*., 2004; Dionisio *et al*., 2005; Yates *et al*., 2006). The plasmid functions responsible for this fitness increase are unknown at present. Apart from systems that directly increase host fitness, plasmids also often encode stabilization functions, including plasmid multimer resolution systems, plasmid partitioning system and various types of postsegregational systems (Bahl *et al*., 2009), which ensure stable plasmid maintenance in the host cell. Another potential maintenance mechanism, even when the plasmid has a detrimental effect on growth, is infectious transfer, where plasmid transmission is efficient enough to overcome negative selection and plasmid loss during host growth. Mathematical modeling has shown that such conditions may exist when conjugative transfer functions are derepressed (at least transiently) and bacterial densities are high (Lundquist & Levin, 1986).

## Conclusions and perspectives

One major conclusion emerging from the past decade or more of studies into antibiotic resistance fitness costs and their relationship with the dynamics of resistance development is that the rate of reversibility in clinical settings will be, at best slow, and possibly, nonexistent. The major reason is that, even if a fitness cost is associated with resistance, compensatory genetic evolution will rapidly reduce this cost and thereby reduce the probability of reversal. In addition, if there is coselection between the resistance mechanism and other selected markers (as there often is for plasmid-borne antibiotic resistance), this will tend to reduce the rate of reversibility driven by fitness costs. This first conclusion could encourage despair. However, a second major conclusion emerging from the recent research into the interplay between resistance and fitness is that we should be able to apply this knowledge to develop strategies to address current resistance problems and to reduce the likelihood of resistance development, in particular for any novel drugs that may be introduced. One obvious possibility is to exploit our expanding knowledge of genomics, physiology and resistance mechanisms, to direct the choice of novel drugs, and drug targets, for which actual or potential resistance

mechanisms would be associated with a high fitness cost and where the expected rate and/or the extent of genetic compensation would be low. A second possibility is to apply our growing understanding of the physiological basis for fitness costs associated with resistance, to design novel therapies, possibly involving drug combinations, to target any Achilles heel associated with a particular resistance mechanism. Finally, the knowledge we are accumulating of the dynamics and mechanisms of antibiotic resistance development should be integrated into mathematical models that can predict the effects of different drug-dosing regimens on efficacy and resistance development. To the extent that we succeed in developing good predictive models, we will have the possibility to shape the outcome of this medically important evolutionary experiment.

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