

Abstract

In recent years, there has been accumulating evidence that antibiotics, besides their antimicrobial action, potentially have a number of undesired side effects that can, at least in some cases, promote genetic variability of bacteria. In addition to resistant variants, antibiotics have also been shown to select mutator clones, thus stimulating evolution towards further resistance. Furthermore, mutations, recombination and horizontal gene transfer have been reported to be somehow affected when bacteria are exposed to subinhibitory concentrations of certain antibiotics. These findings may have implications for the use of antibiotics, because they may have undesired side effects, such as enhancing antibiotic resistance evolution. Here we present data supporting (or not) this fearsome possibility and discuss whether this potential threat should be taken into consideration.

Introduction

“What does not kill me, makes me stronger.”

(Friedrich Nietzsche, Twilight of the Idols, 1889).

The beginning of mass production of antibiotics over half a century ago represented a major breakthrough in the medical treatment of infectious diseases. Physicians and the scientific community were so enthusiastic about their initial success in fighting pathogens that in 1970, the US Surgeon General stated that the war against pestilence had been won (Anker & Schaaf, 2000). After antibiotics were first introduced, the microbiologists were so confident that they assumed that evolution of antibiotic resistance was unlikely because the frequency of mutation to resistance in bacteria was negligible (Davies, 1994).

Unfortunately, time has proved that they were overoptimistic. Nobody could anticipate the wide variety of mechanisms the bacteria use to evolve, especially their amazing ability of interchanging genes, which is now well known as horizontal gene transfer (HGT). This unexpected capacity of microorganisms is the reason why, in spite of the success of antibiotics in curing a considerable number of infectious diseases, the emergence of multidrug-resistant bacteria is currently a major worldwide concern, because it is the major cause of treatment failure against many pathogens (Hoiby, 2000). It is now clear that the extended use of antibiotics over the past six decades has led to the selection and spread of resistant bacteria. In fact, the emergence of resistant bacteria can be considered one of the most rapid and striking phenomena of biological evolution generated by mankind (Blazquez, 2003).

The evolution of antibiotic resistance is, obviously, based on genetic variation and selection of the genotypes generated by this variation. The classical view of this issue holds that the exposure of bacteria to antibacterial agents results in the selection of pre-existing resistant variants that ultimately become fixed in the population (Luria & Delbruck, 1943; Newcombe, 1949; Lederberg & Lederberg, 1952). After antibiotics were first introduced, the microbiologists were so confident that they assumed that evolution of antibiotic resistance was unlikely because the frequency of mutation to resistance in bacteria was negligible (Davies, 1994).

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pathogens, and also of commensal strains (known to be a major reservoir of resistance), to antibiotics and host defences.

However, favouring mutator clones that are prone to develop antibiotic resistance is not the only detrimental side effect that antibiotic therapy might produce. It has been known for a long time that sublethal concentrations of many antibiotics can interfere with some important aspects of bacterial physiology, and these interferences may have undesirable outcomes in some cases (Lorian, 1975; Goh et al., 2002; Davies et al., 2006). The reported effects of these sublethal concentrations include changes in the morphology, virulence and ability to produce genetic variation. The scope of this review is limited only to the latter.

These phenomena may be counterintuitive at first glance, as one could naively expect the minimal inhibitory concentration (MIC) of an antibiotic to be a strict threshold in an ‘all or nothing’ behaviour. Nevertheless, antibiotics in many cases have shown a biphasic dose–response nature, which is actually recognized as a typical case of hormesis (Calabrese & Baldwin, 2002; Davies et al., 2006). Hormesis is a Helenistic Greek term used to define the phenomenon by which an agent has different and even opposite effects at high and low concentrations. An example of this hormetic behaviour is found in quinolones, which disrupt DNA replication and are lethal at high doses, but promote exotoxin secretion at low ones, as observed in enterohaemorrhagic Escherichia coli O157:H7 (Yoh et al., 1999). There is currently some controversy about the biological meaning of this hormetic effect of antibiotics. For some authors, this is reminiscent of their ecological role as signalling agents (Davies et al., 2006; Linares et al., 2006), while for others this is just the result of interference with the metabolic and transcriptional networks that turn on general stress responses (Huffman et al., 2005; Blazquez et al., 2006). Here, we would like to emphasize that these two interpretations, if true, are not necessarily exclusive, although more research is still required.

Apart from academic interest, these hormetic effects of antibiotics may represent a new threat as they could, at least in some cases, induce some virulence determinants and promote evolution of both pathogens and commensals via stimulation of genetic variability. Together with the promotion of mutator clones, these findings have implications for the use of antibiotics, because nonlethal concentrations can enhance virulence and resistance evolution. Our review focuses mainly on the evolutionary implications, with possible clinical and ecological consequences, that this phenomenon might have. Other reviews on experimental studies of the unintended side effects of antibiotics can be found elsewhere (Lorian, 1975; Goh et al., 2002; Hastings et al., 2004; Davies et al., 2006).

In this review, we discuss briefly the current knowledge of the effects of antibiotics on genome stability. These effects may influence the evolution and spread of resistance determinants by different phenomena, including selecting mutator clones and enhancing mutation, HGT or recombination. This indicates that some antibiotics may have the undesirable effect of enhancing the emergence and spread of antibiotic resistance. In the end, we discuss to what extent the design of therapy should consider this potential threat, and suggest future research priorities.

Antibiotic selection of evolution-prone bacteria

Seminal studies have demonstrated that the exposure of bacteria to antibacterial agents results in the selection of pre-existing resistant variants that ultimately become fixed in the population (Luria & Delbruck, 1943; Newcombe, 1949; Lederberg & Lederberg, 1952). In addition, antibiotic pressure may also select for cells with an increased frequency of mutation (hypermutators) (Mao et al., 1997), which, in many cases, also have an increased frequency of recombination of divergent sequences (Rayssiguier et al., 1989; Mao et al., 1997; Taddei et al., 1997; Shaver et al., 2002; Blazquez, 2003). This selection occurs because mutator clones have a higher probability of producing mutants that survive the strong selection imposed by antibiotics, and thus their frequency increases by means of a process known as ‘genetic hitch-hiking’: the rise of a mutator allele because it keeps on linked to the beneficial mutations it has caused (Mao et al., 1997; Taddei et al., 1997; Shaver et al., 2002). This phenomenon may lead to an unpleasant scenario in which, in some chronic infections, as populations of bacteria face successive antibiotic administrations, they are increasingly likely to develop further antibiotic resistance due to an increased mutation rate. This was dramatically illustrated by Oliver et al. (2000), who demonstrated that Pseudomonas aeruginosa, after years of treatment with antibiotics in cystic fibrosis patients, became highly resistant to a huge number of antibiotics. On a broader scope, Denamur et al. (2005) have recently shown that there is a significant correlation between having moderate mutation frequencies and having multiple antibiotic resistances.

Antibiotic-induced mutagenesis

The classical view states that bacteria are passive agents on their adaptive process, and for a long time it has been assumed that antibiotic treatments select for pre-existing ‘lucky’ antibiotic-resistant variants. However, accumulating evidence suggests that bacteria are not simply passive subjects along their process of evolution by mutation and natural selection, as they have mechanisms that promote genetic variation under stress. Besides selecting hypermutable strains, antibiotics have been suggested to enhance mutations conferring antibiotic resistance via induction of transient mutagenic mechanisms (Rosenberg, 2001).
Antibiotic-induced mutagenesis presumably occurs because of the production of single-stranded DNA and therefore induction of the SOS response (see Fig. 1).

The *E. coli* SOS system comprises at least 30 different genes, some of them encoding proteins involved in DNA repair pathways (Fernandez De Henestrosa et al., 2000). When activated by single-strand DNA (which can be created by multiple events, such as DNA damage, stalled replication, conjugation, etc.), the coprotease activity of the RecA protein promotes the self-cleavage of the LexA protein (the SOS repressor) and, consequently, triggers the SOS response (Friedberg et al., 2006).

The genes upregulated by the SOS response include *recA* and *lexA* and those encoding the error-prone DNA polymerases Pol-II, DinB (Pol-IV) and UmuCD (Pol-V), which ultimately are responsible for introducing the mutations (Walker, 1995; Napolitano et al., 2000). Whether SOS mutagenesis is exclusively a byproduct of the cellular capacity to cope with damaged DNA or is a selected product through evolution is a topic for future studies and is beyond the scope of this short review. What is clear is that even if error-prone DNA polymerases do not evolve to speed up evolution, they may accelerate it.

In this respect, quinolones, a widely used family of DNA-damaging antibiotics, have been known for years to be mutagenic in bacteria (Ysern et al., 1990; Gocke, 1991). These antibiotics exert their action by inhibiting DNA gyrase, which causes replication fork to stall, thereby producing single-stranded DNA and SOS induction (Gocke, 1991). A number of studies have shown that subinhibitory concentrations of quinolones rise, as expected, the frequency of resistance mutants in some bacteria. In *Streptococcus pneumoniae*, for instance, exposure to ciprofloxacin produces up to fivefold increase in the frequency of rifampin-resistant mutants (Henderson-Begg et al., 2006). Also, ciprofloxacin has been shown to enhance the yield of carbanapem resistance in *P. aeruginosa* in about the same order (Tanimoto et al., 2008). Finally, a more dramatic effect resulted from ciprofloxacin usage on *Mycobacterium fortuitum*, with an increase in the mutation frequency almost two orders of magnitude (Gillespie et al., 2005).

Recently, it has been shown that the challenge of *P. aeruginosa* with sublethal concentrations of the cephalosporin ceftazidime, an inhibitor of cell wall synthesis, elicits a plethora of adaptive responses including increases in the mutation frequency (Blazquez et al., 2006). β-Lactam antibiotics have also been demonstrated recently to be good inducers of the SOS response (Miller et al., 2004; Perez-Capilla et al., 2005). However, in *E. coli*, the ceftazidime-mediated induction of mutation, which proceeds via dinB overexpression, is surprisingly independent of the SOS response (Perez-Capilla et al., 2005). The observation of Cortes et al. (2008), of up to a threefold increase in the mutation rate in *S. pneumoniae* after the exposure to subinhibitory concentrations of penicillin, may also be interpreted in this way.

**Antibiotic-induced lateral transfer and recombination**

Apart from mutation, there are other mechanisms that induce heritable variation in bacteria: (1) intragenomic reshuffling of genomic sequences (intrachromosomal recombination) and (2) the acquisition of DNA sequences from other organisms via HGT. Both mechanisms play a major role in pathogen evolution, allowing bacteria to evade the immune response, distributing genes that increase virulence or conferring increased resistance to antibiotics (Guttman & Dykhuizen, 1994; Lawrence & Roth, 1996; Lawrence & Ochman, 1998; de la Cruz & Davies, 2000). As the available sequence data from different species strongly...
suggest, the majority of antibiotic resistances have most likely been acquired through the lateral transfer of resistance genes from other ecologically and taxonomically distant bacteria (Binnewies et al., 2006). In addition, it has been estimated that any single nucleotide change is about 50 times more likely to have occurred by recombination than by a de novo mutation for E. coli in nature (Guttman & Dykhuizen, 1994).

SOS-inducing antibiotics have received considerable attention in recent years not only for their capacity to increase mutagenesis but also because they have been described to promote the transfer of mobile genetic elements in some species. In E. coli H57:0157, for instance, Zhang et al. (2000) showed that treatment with fluoroquinolones triggers, via SOS, the expression of the genes of a prophage harbouring, among others, a gene coding for the Shiga toxin. This has alarming implications, as fluoroquinolones are commonly used to treat the shiga toxin-dependent haemolytic–uraemic syndrome, and this SOS induction not only worsens the syndrome by overproduction of the toxin but also promotes the excision and transfer of the prophage, thus amplifying the population of phages encoding the Shiga toxin (Wong et al., 2000).

Moreover, SOS induction, as a result of antibiotic-induced host damage, activates mobilization of many other mobile elements. In Vibrio cholerae, as an example, Beaber et al. (2004) observed that an integrating conjugative element, encoding resistance to chloramphenicol, sulphamethoxazole, trimethoprim and streptomycin, was transferred and integrated with much higher efficiency when the donor was exposed to concentrations below the MICs (sub-MICs) of two DNA-damaging antibiotics: mitomycin C and ciprofloxacin. They showed that induction of SOS response by DNA damage is responsible for upregulating the transcriptional activators required for excision and transfer (Beaber et al., 2004).

In another work, Ubeda et al. (2005) have recently reported that SOS induction by low doses of ciprofloxacin promotes the transference of pathogenicity island-encoded virulence factors in Staphylococcus aureus. They convincingly show that the pathogenicity island SaPibov1 is mobilized after SOS induction of some different temperate phages and is then packaged into phage-like particles and transferred at a high frequency (Ubeda et al., 2005). Other mobile genetic elements have also been observed to increase their transfer frequency under the challenge of non-SOS-inducing antibiotics. Stevens et al. (1993) showed that a 100-fold increase in gene transfer was induced by exposure to low concentrations of tetracycline in Bacteroides strains harbouring conjugative transposons (all encoding tetracycline resistance). Similar enhancing effects of this antibiotic have been observed for conjugative transposons of other bacterial genera such as Enterococcus faecalis (Torres et al., 1991). Another case of SOS-independent antibiotic induction of horizontal transfer was reported about two decades ago. Barr et al. (1986) showed that exposure of S. aureus cultures to subinhibitory concentrations of some β-lactams increased the transfer of a plasmid with tetracycline resistance by a factor of 100–1000-fold. They suggested that this phenomenon was probably due to the formation of large bacterial aggregates (Barr et al., 1986). More recently, (Prudhomme et al., 2006) showed that of some antibiotics are capable of inducing DNA transformation, via competence, in the naturally competent S. pneumoniae. They demonstrated that sub-MICs of different antibiotics induced transcriptional activation of the com regulon, which comprises a set of genes related to DNA uptake and the capacity to kill noncompetent cells. Although the specific molecular mechanism remains unknown, it presumably proceeds in an SOS-independent manner, as S. pneumoniae lacks any SOS-like system (Prudhomme et al., 2006).

Besides lateral transfer, recombination has been shown to increase under antibiotic stress. Lopez et al. (2007) have recently described ciprofloxacin as a stimulator of intrachromosomal genetic recombination (recombination of DNA sequences from the same chromosome) of both identical and divergent DNA sequences in E. coli. As discussed previously, quinolones have long been known to induce SOS by producing single-stranded DNA. This result should not be surprising as high levels of RecA are known to increase the recombination frequency between divergent sequences (Dimpfl & Echols, 1989; Matic et al., 1995). In fact, it has been suggested that the prevention of LexA cleavage during antibiotic treatment may render bacteria unable to develop antibiotic resistance (Cirz et al., 2005). However, the most striking result of the work from Lopez et al. (2007) is that this ciprofloxacin-stimulated recombination is not dependent on SOS induction, as this stimulation also occurs in a mutant carrying a cleavage-resistant LexA protein and hence cannot trigger the SOS response. Consequently, they suggested to inhibit RecA rather than LexA to prevent both the antibiotic resistance development produced via mutation and the spread and improvement via recombination (Lopez et al., 2007). Intrachromosomal recombination may provide shortcuts to adaptation by allowing the reshuffling between single mutants, in contrast to the time-consuming serial accumulation of successive mutations (Crameri et al., 1998). A paradigmatic example could be the case of extended-spectrum β-lactamases, which have been reported to be the result of combining a reduced number of mutations (Blazquez et al., 1995, 2000).

Despite all these data illustrating the capacity of low doses of some antibiotics to enhance mutation, recombination and HGT, not all the described side effects of antibiotics imply heightened evolvability, defined as the capacity to generate the selectable variation needed to respond to
selection pressure (Houle, 1992). Interestingly, the opposite effect has also been reported. It has been known for decades that some antibiotics, among a wide variety of chemicals, can inhibit conjugation, show preferential toxicity against plasmid-bearing cells or stimulate plasmid curing (Viljanen & Boratynski, 1991). For instance, ciprofloxacin can impede R-plasmid transfer by inhibiting conjugation in *E. coli* (Weisser & Wiedemann, 1987). Another example can be found in Al-Masaudi *et al.* (1991), where sub-MICs of the antibiotic mupirocin have been shown to reduce the conjugal transfer of the *S. aureus* gentamicin resistance plasmid pWG613 by >1000-fold.

**Are those phenomena biologically and clinically relevant?**

According to the recent studies discussed here, it seems that a frightening panorama is emerging: antibiotic therapy might lead to the unfortunate outcome of generating more hazardous pathogens with increased virulence and resistance levels. It is worth noting that, to date, it remains to be proven to what extent such effects play an important role in the final emergence of clinically relevant virulent and antibiotic-resistant mutants in naturally occurring bacterial populations.

There are at least three open questions regarding this issue. First, can bacteria take advantage of the genome instabilities caused by antibiotics? Achieving this implies that a significant part of the population must be viable at the appropriate antibiotic doses to produce genetic variability. Note that to be effective, the production of variability must occur under nonlethal concentrations, i.e. although sublethal, antibiotic doses have to be close to lethal, because lower concentrations will not induce the transient variability and higher concentrations will kill most of the cells in the population. Furthermore, to be relevant, the size of this subpopulation must be large enough not to offset the increase in variability produced by the treatment. This is because the total variation income of a population (novelty supply rate; see Fig. 2) depends on both population size and genetic instability. In other words, a drastic increase in mutation or recombination rates will be of no use if the concentration of antibiotic required is such that only a small number of cells will survive. Some studies indicate that bacteria can easily recover after a challenge with these kinds of concentrations (Blazquez *et al.*, 2006; Lopez *et al.*, 2007).

![Fig. 2.](image)

**Fig. 2.** Theoretical example of undesirable antibiotic side effects on genetic variability. To illustrate how the stimulation of both mutation and recombination can affect evolution, it may be useful to introduce the concept of the ‘novelty supply rate’, which can be defined as the amount of genetic variation supplied in each generation. It could be simply defined as the product of population size and the mutation and recombination rates, \( v = N(\mu + r) \) (where \( N \) is the population size and \( \mu \) and \( r \) the mutation and the recombination rate, respectively). Adaptive evolution will be a function of this value. (a) Effect of increasing concentrations of an antibiotic on \( v \), using realistic values for \( N, \mu \) and \( r \) (see Lopez *et al.*, 2007). The left axis indicates the population size (expressed as the ratio between the surviving population and the initial one). The right axis indicates the theoretical increase (fold) of novelty supply by mutation, recombination or both. Black line (left axis), decrease in population size upon antibiotic treatment; green line (right axis), mutation rate; blue line (right axis), recombination rate; and red line (right axis), novelty supply. The shaded area covers the antibiotic concentrations for which this effect has been reported. This area represents a stress-induced adaptive window where the supply of variability is increased. Note that even though the population size has been reduced, the novelty supply rate has increased several times beyond its untreated value, thus increasing the probability of the remaining population becoming resistant to the current or any other antibiotic. However, whether or not this phenomenon could have adverse implications relies very much on the size of the surviving subpopulation. For instance, more dramatic reductions of population size would counteract these undesirable effects of antibiotics on genetic instability (b).
The second question is what is the probability of a specific bacterial population to face such a narrow interval of subinhibitory concentrations (the stress-induced adaptive window of Fig. 2). Variability of susceptibility is expected not only between species but also within each species, so that the interval may be not so narrow (Zhanel et al., 2002). Moreover, owing to different factors, a huge diversity of spatial and temporal antibiotic concentration gradients may occur in the human body (Baquero et al., 1998). Furthermore, even antibiotic-resistant bacteria may find the mentioned window, but at higher concentrations. This is because a higher level of resistance to a specific antibiotic implies a change in the stimulating window’s position, but not its elimination. Therefore, any particular window of stimulating antibiotic concentrations should not be difficult to find. Finally, although antibiotics are mainly used to combat pathogens, they also challenge commensals collaterally. While an infection is usually caused by a relatively small number of cells (10^6–10^7), about 10^14 prokaryotic cells belonging to hundreds of different species conform our commensal flora (Andremont, 2003) and these species have different intrinsic levels of antibiotic susceptibility (Zhanel et al., 2002). The fact that thousands of tons of different antibiotics are used every year to treat billions of human and veterinary infections and to promote animal growth increases the probability of finding virtually any suitable condition for stimulating recombination (Wise et al., 1998).

The third question that could be asked is as follows: is the effect of a magnitude sufficient to somehow influence bacterial evolution? Among the works reported above, there are considerable differences in the degree of stimulation of each particular trait. However, it would be almost impossible to answer this question properly without conducting well-planned in vivo studies. In spite of this, it is our view that even modest effects should be considered because nothing is black or white in nature, only shades of grey, and small differences sometimes draw a fine dividing line between failure and success. For instance, modest changes in mutation frequency have been shown to influence antibiotic resistance development (Denamur et al., 2005; Orlen & Hughes, 2006). Likewise, modest increases in recombination or HGT may have similar effects.

**Concluding remarks**

Throughout this review, we have presented the available data showing that antibiotics can favour the selection of evolution-prone bacteria and that some antibiotics can affect genome stability at sublethal concentrations. Mutagenicity, increased DNA transfer from treated bacteria, increased genetic transformability and increased genetic recombination of both identical and divergent DNA sequences have been reported to be somehow affected when recipient bacteria are exposed to subinhibitory concentrations of certain antibiotics. This indicates that certain virulent organisms that survive exposure to antibiotics may become more resistant, by point mutation, acquisition of foreign DNA and recombination or reshuffling of their own genetic information.

Horizontal transfer, mutation and recombination play important separate roles in the acquisition of antibiotic resistance, but they also act synergistically. Horizontal transfer introduces new genes and alleles into a population, and mutation produces new variations by introducing single mutations in them. These single mutations can be rearranged together by recombination, and these new alleles, in turn, could be transferred again, restarting the synergistic cycle. This seems to be the case for some antibiotic resistance genes, such as β-lactamase enzymes, that are known to be widespread among several species and to exhibit a huge single-mutation degree of polymorphism (Blazquez et al., 1995, 2000; Crameri et al., 1998).

Obviously, more in vitro and in vivo research is needed to expand our knowledge of the effect of antibiotics on bacterial evolvability. However, the time has come to realize that antibiotics are selectors not only of resistant bacteria but also true promoters of antibiotic resistance.

Following the above considerations, it would be desirable that the design of therapy should consider these potentially risky side effects of antibiotics. The same applies for the launch of new antibiotics. We also hope that the results reviewed here would help promote the use of antibiotics in a more restrained and conscientious way.

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**References**


