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Antibiotic-Induced Genetic Variation: How It Arises and How It Can Be Prevented

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Abstract

By targeting essential cellular processes, antibiotics provoke metabolic perturbations and induce stress responses and genetic variation in bacteria. Here we review current knowledge of the mechanisms by which these molecules generate genetic instability. They include production of reactive oxygen species, as well as induction of the stress response regulons, which lead to enhancement of mutation and recombination rates and modulation of horizontal gene transfer. All these phenomena influence the evolution and spread of antibiotic resistance. The use of strategies to stop or decrease the generation of resistant variants is also discussed.

Contents

In life, unlike chess, the game continues after checkmate.

—Isaac Asimov (1920–1992)

INTRODUCTION

The beginning of mass production of antibiotics over half a century ago was a major breakthrough in medical treatment of infectious diseases and was the advent of modern medical practices, including complicated surgical interventions and cancer treatments (see the sidebar titled Note). Without effective antibiotics, modern medicine is simply unfeasible. For several generations, we have lived in a comfortable era in which most bacterial infections have been resolved successfully with antimicrobial drugs. As a result of the extensive use and misuse of antibiotics in human and veterinary medicine and in agriculture (1, 25, 104, 158), the tremendous emergence and spread of antibiotic resistance among bacterial pathogens in recent decades has threatened this idyllic world. The problem has been exacerbated by waning pharmaceutical investment in research for new antimicrobial drugs. There is thus an urgent need to take measures to avoid returning to the preantibiotic era (6). The development of new antibiotics, the implementation of methods to increase treatment efficiency, and a better understanding of the impact of antibiotics on bacteria and their responses are among these necessary measures (73).

NOTE

According to Waksman, an antibiotic is a chemical substance produced by microorganisms with the capacity to inhibit growth of and destroy bacteria and other microorganisms (154). Nonetheless, some synthetic substances such as quinolones, sulphonamides, and oxazolidinones (defined as antimicrobials elsewhere) have the same activity profiles but are not encompassed by this definition. For the sake of simplicity, in this review we will use both terms without distinction.

The concurrence of genetic variation and natural selection is the most fundamental force in biology. Seminal studies demonstrated that exposure of bacteria to effective concentrations of antibacterial agents, including bacteriophages, selects for preexisting resistant variants that eventually become fixed in the population (115). The evolution of antibiotic resistance is obviously based on genetic variation and selection of the resistant variants. It has nonetheless become clear that antibiotics do more than simply select for resistant variants. At subinhibitory concentrations, they induce bacterial responses, including mechanisms that accelerate the adaptation rate (22, 50, 138). Here it is important to highlight that the inhibitory effect of an antibiotic is not only related to the intrinsic susceptibility of a strain, antibiotic concentration, or a specific environment; time of exposure is also relevant to antibiotic activity. Exposure to high antibiotic concentrations for a very short period can have the same effect as challenges with subinhibitory concentrations for longer times. Here we therefore consider subinhibitory concentrations to be those that do not stop growth or kill the challenged bacteria, independently of drug concentration.

Subinhibitory concentrations: concentrations that do not stop growth or kill the challenged bacteria; experimental conditions must be defined when a value is provided

From a clinical perspective, it has been assumed that only antibiotic resistance levels above a defined threshold (related to antibiotic concentration at the site of infection as well as strain susceptibility) are relevant for evolution of resistance. For this reason, the impact of subinhibitory antibiotic concentrations on bacterial evolution to resistance has long been neglected, although it was recognized as an important step on the selective paths to strong resistance (15). Recent information indicates that very low antibiotic concentrations can select not only for low-level resistance (12) but also for high-level resistance (65).

Bacteria frequently encounter low concentrations of antibiotics in their environment. When antibiotics are used therapeutically or for growth promotion, for example, bacterial populations face wide gradients of antibiotic concentrations in compartmentalized animal or human bodies. These gradients are due to pharmacokinetic factors such as different diffusion rates into various cell or tissue types, metabolism, local binding or inactivation, or variation in the elimination rate from different body compartments (15). The direct effect of normal or pathogenic microbiota, particularly if they possess antibiotic-inactivating enzymes, can also contribute to gradient formation. Because antibiotics are poorly absorbed by the gut, 30–90% of ingested antibiotics are excreted unchanged via urine and feces from human and animal bodies (see for example Reference 128). Some antibiotics have high persistence in the environment; for example, azithromycin and amoxicillin are commonly found in soil and water (82, 84). Some molecules such as sulfamethoxazole, erythromycin, and the antifolate trimethoprim are even more concentrated after wastewater treatment due to deconjugation of their metabolites, which restores the parent molecules (130). Fluoroquinolone concentration can be particularly high in dry mass of farmyard manure and of sewage sludge (63). Finally, antibiotics are also excreted by antibiotic-producing microorganisms in all environments. The omnipresent low doses of antibiotics in the environment, together with their major impact on bacterial homeostasis and genetic stability, set the perfect scenario for antibiotics to have an important role in the evolution of antibiotic resistance (5, 103).

In this review, we discuss current knowledge of how antibiotics cause bacterial genetic instability and thus influence the evolution and spread of resistance determinants in different ways, including enhancement of mutation and recombination, horizontal gene transfer (HGT), and promotion of adaptive responses. These last two processes will not be treated here, however, as they have been covered extensively in several fine reviews on antibiotic side effects (5, 41, 59, 67, 75, 98). Finally, we discuss the possibility of using strategies to halt or reduce the generation of resistant variants.

ANTIBIOTIC-INDUCED MUTAGENESIS

Mutations can contribute to antibiotic resistance in a number of ways. They can confer resistance by modifying genes coding for the antibiotic targets, attenuate fitness reduction associated

with acquired antibiotic resistance, or modify activity and the resistance spectrum of horizontally transferred resistance determinants. Indeed, even a slight increase in mutation rates contributes significantly to the evolution of antibiotic resistance (40, 42, 43, 121). Spontaneous mutation rates are maintained at low levels by a multitude of molecular mechanisms that include metabolic control over concentrations of endogenous and exogenous mutagens, DNA repair systems, dNTP pool balance, base selection and proofreading activity of replicative DNA polymerases, and several postreplication systems for repairing mismatches (106). When normal cell function is disturbed by environmental factors that reduce fitness and/or increase mortality, mutation rates also commonly increase. Antibiotics are among the environmental factors known to increase mutation rates (5, 22, 88); they induce mutagenesis during exposure of bacterial populations to lethal concentrations of antibiotics, but also during exposure to subinhibitory concentrations in many species, including *Acinetobacter baumannii*, *Enterobacter cloacae*, *Escherichia coli*, *Mycobacterium fortuitum*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella enterica* Typhimurium, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Vibrio cholerae* (7, 11, 24, 30, 45, 54, 56, 57, 66, 68, 81, 86, 88, 92, 96, 126, 134, 137, 147–149, 152).

Low doses of antibiotics, both bactericidal and bacteriostatic, with very different cellular targets increase mutagenesis in bacteria (11, 149). It is not surprising that antibiotics like trimethoprim and the fluoroquinolone ciprofloxacin, which directly affect DNA replication and genome integrity, can increase mutation rates (57, 147, 153). What was not anticipated, however, is that many antibiotics that do not affect DNA directly, such as the β-lactam ampicillin or the aminoglycoside streptomycin (which interfere respectively with synthesis of the peptidoglycan layer and with protein synthesis), also increase mutation rates (66, 134). These observations suggest that antibiotics can affect mutation rates by a wide variety of mechanisms. Current knowledge of the mechanisms of antibiotic-induced mutagenesis indicates that they can be classified in three largely overlapping groups: oxidative metabolism and the SOS and the RpoS-regulated general stress responses (**Figure 1**).

Oxidative Metabolism

Oxidative metabolism is a process in which oxygen is used to generate energy from nutrients. In bacteria growing in aerobic conditions, most energy is produced by respiration using molecular oxygen as the final electron acceptor, although unwanted by-products of respiration are reactive oxygen species (ROS). The major source of endogenous ROS production is accidental autoxidation of respiratory enzymes and electron leakage (78). ROS are formed continuously but are also eliminated continuously by superoxide dismutase transformation of superoxide to hydrogen peroxide, and by peroxidases and catalases that degrade hydrogen peroxide. Hydrogen peroxide is a liability for the cell, as its reduction by redox-active metals such as iron produces highly reactive hydroxyl radicals that cannot be eliminated by an enzymatic reaction. These radicals are very toxic since they react with all cell macromolecules: nucleic acids, amino acids, carbohydrates, and lipids. Bacteria have defense mechanisms just sufficient to protect themselves against endogenous ROS. For instance, *E. coli* superoxide dismutase mutants and *fur* mutants, which have elevated intracellular iron content, show high spontaneous mutation rates (78). A stress-induced increase in ROS production thus has deleterious consequences, including increased mutation rates. Some studies have reported that treatment with ampicillin, kanamycin, norfloxacin, ciprofloxacin, or trimethoprim increases ROS production and mutation rates in *E. coli* (57, 62, 86, 149).

This increase in mutagenesis can be prevented by the ROS scavenger thiourea (113), by anaerobic conditions, or by inactivating the *tonB* gene, which reduces extracellular iron importation and thus lowers hydroxyl radical production (57). Levels of 8-oxo-deoxyguanosine (8-oxo-dG), the most mutagenic ROS-oxidized molecule, increase in the DNA of ampicillin-,

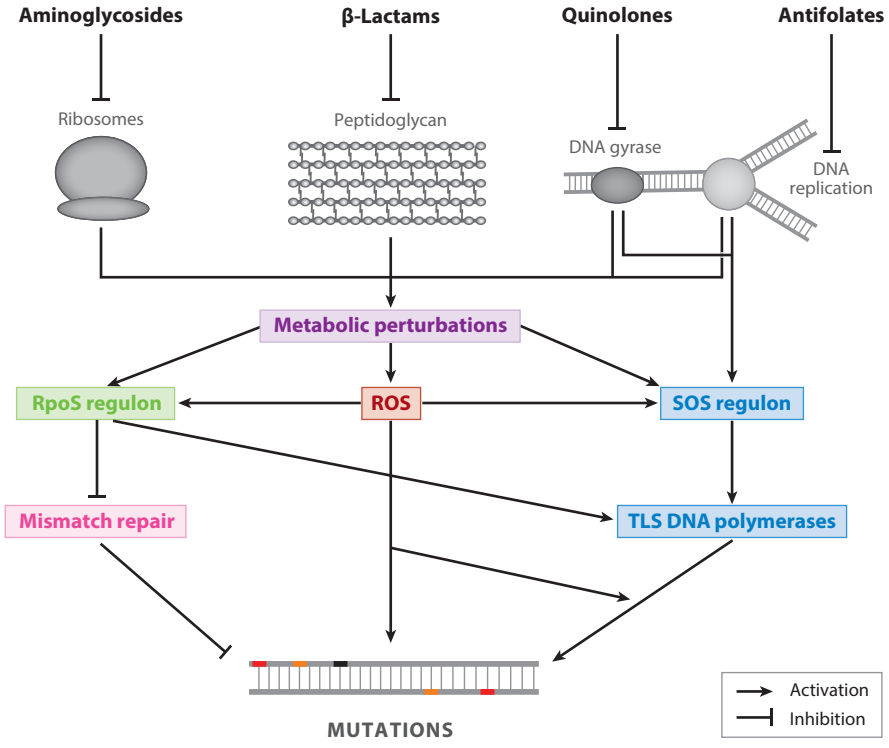

Figure 1

How antibiotics modulate mutation rates in bacterial cells. Different antibiotics have distinct cellular targets: quinolones and antifolates block DNA replication, β-lactams affect peptidoglycan synthesis, and aminoglycosides perturb protein synthesis. Treatment with DNA replication inhibitors increases mutagenesis, as they induce SOS-controlled, error-prone translesion synthesis (TLS) DNA polymerases (*blue*). Other antibiotics, which do not target DNA, increase mutation rates because they cause metabolic perturbations (*purple*). Antibiotic-treated cells increase energy production, as well as translation and macromolecular repair and protection capacity, to control antibiotic-induced damage and restore growth. Increased energy requirements need cell respiratory activity, which results in increased ROS production (*red*). ROS damage DNA and hence contribute to increased mutagenesis. Elevated energy production also causes accelerated nutrient depletion that in turn triggers RpoS regulon (*green*) induction. Induction of this regulon results in increased mutagenesis, since it inhibits activity of the mismatch repair system (*magenta*), a major guardian of genetic stability, and because it stimulates activity of the TLS DNA polymerases. Abbreviation: ROS, reactive oxygen species.

kanamycin- or norfloxacin-treated cells compared to untreated *E. coli* cells (18); ampicillin-induced mutation rates are suppressed by catalase overexpression in *E. coli* (81). Mutations induced by subinhibitory concentrations of streptomycin, which also increase translation errors, are predominantly A:T to T:A and G:C to T:A transversions, both hallmarks of oxidative DNA damage (106). ROS are also good SOS inducers, as they damage DNA directly (87). In addition, protein mistranslation caused by oxidative damage leads to the collapse of replication forks, which induces the SOS response (2). ROS therefore contribute cumulatively to the mutagenic effect of antibiotics (see below). In addition, ROS appear to be major contributors to the ciprofloxacin-induced SOS response and mutagenesis in *E. coli* (A. Rodriguez-Rosado, personal communication).

Respiratory activity increases in antibiotic-treated (stressed) cells, giving rise to higher ROS levels (47, 95). Stressed cells probably increase respiratory activity because they need more energy

to repair macromolecular damage and restore growth. For example, ATP levels increase in ultraviolet- and nalidixic acid–treated *E. coli* cells; levels drop when DNA lesions are repaired in wild-type cells but remain high in *recA* mutants, which cannot repair DNA lesions (16, 38). In addition, antibiotic-treated cells have increased translational capacity, which is also very energy-consuming (103). ROS cannot be produced in the absence of oxygen. In anaerobic conditions, however, nitrate respiration contributes to ampicillin, gentamicin, norfloxacin, and trimethoprim cytotoxicity in *E. coli* (47, 57). It could thus be hypothesized that antibiotics increase mutagenesis through anaerobic respiration–mediated production of reactive species. Nonetheless, some studies detected no ROS signatures in ciprofloxacin- or norfloxacin-mediated mutations in *E. coli* (96, 147).

The SOS Response

Antibiotics belonging to different classes induce the SOS response in distinct gram-positive and -negative bacterial species (10, 11, 58, 93, 101, 105, 145, 162, 163). The SOS response is a coordinated cellular response to DNA damage and replication blockage. During normal growth, the LexA protein represses genes of the SOS regulon. The SOS response inducer is single-stranded DNA (ssDNA) when it accumulates at above-physiological levels. Contact with ssDNA activates RecA protein coprotease activity, which promotes the proteolytic autocleavage LexA, thus activating the SOS response. The relative strength of LexA binding to its promoters determines the sequential order of gene induction during the SOS response. Once DNA damage is repaired, DNA replication is restarted, ssDNA disappears, and the SOS regulon is re-repressed. In *E. coli*, the SOS regulon is composed of at least 40 genes, many of which code for DNA repair functions such as nucleotide excision repair, translesion synthesis (TLS), and homologous recombination (144). SOS regulons are found in many other bacterial species, with conserved LexA/RecA regulatory core and variable gene content (48). Antibiotic-induced mutagenesis can therefore be considered SOS dependent, as it requires functional RecA and LexA regulatory proteins. Based on this criterion, a variety of antibiotics from different families induce SOS-dependent mutagenesis in *E. coli*, *P. aeruginosa*, *S. aureus*, and *V. cholerae* (11, 71, 111, 112, 149).

Antibiotics induce SOS by a variety of molecular mechanisms; for example, dTTP depletion by trimethoprim blocks DNA replication (57, 139), SOS induction by β-lactams involves the DpiBA two-component system in *E. coli* (105), and SOS induction by tobramycin is triggered by RNA polymerase stalling in *V. cholerae* (9). Antibiotic-induced SOS-dependent mutagenesis relies on a single group of terminal effectors, the Y family TLS DNA polymerases. Inactivation of TLS polymerase–encoding genes abolishes antibiotic-induced mutagenesis in *A. baumannii*, *E. coli*, *P. aeruginosa*, and *V. cholerae* (7, 30, 66, 124).

The common characteristic of TLS polymerases is the ability to by-pass DNA lesions that block replicative DNA polymerases (151). TLS polymerases are inherently error prone, as they by-pass noncoding lesions and have no proofreading activity. Overexpression of genes coding for TLS polymerases that results from induction of the SOS regulon can be sufficient to increase mutagenesis. The propensity of the TLS polymerases to incorporate mutagenic oxidized nucleotides into DNA might also be responsible for antibiotic-induced mutagenesis associated with oxidative stress (51, 160).

E. coli has two Y family TLS polymerases, pol IV and pol V, encoded by the *dinB* and *umuCD* genes, respectively (116). Chromosomes of most bacterial species have homologs of these two DNA polymerases, which are also carried by naturally occurring plasmids and integrating conjugative elements (79, 151). Because TLS polymerases encoded by plasmid-borne genes are very error prone, Ames and colleagues (92) introduced R-plasmid pKM101, which encodes the pol V orthologs *mucAB* in *S.* Typhimurium tester strains developed to identify potential mutagens and

carcinogens. MucAB greatly increases fluoroquinolone-induced mutagenesis in *S.* Typhimurium (163).

Other results show antibiotic-mediated mutagenesis independent of TLS polymerases. For instance, some ciprofloxacin-induced genetic changes in *E. coli*, such as deletions, do not require any SOS-induced polymerase (147). A study by Pomerantz et al. (129) suggests the participation of DNA pol I in this TLS-independent mutagenesis. Pol I normally exhibits high-fidelity DNA synthesis but is very error prone at the RecA-mediated D-loops produced by double-strand break repair (129). The double-strand break produced by some antibiotics such as ciprofloxacin can generate these RecA-mediated D-loops and cause subsequent TLS-independent mutagenesis.

Sigma (σ) factors: proteins that enable specific binding of RNA polymerase to gene promoters to initiate transcription; they provide effective mechanisms for regulating large numbers of genes

The RpoS-Regulated General Stress Response

The general stress response regulon, which is controlled by the alternative RNA polymerase sigma factor RpoS (σ^S), is conserved in many bacterial species (28). This regulon comprises hundreds of functionally unrelated genes and can be induced by a wide range of stress conditions, including nutrient starvation, heat shock, low pH, and oxidative or osmotic shock, as well as bactericidal and bacteriostatic antibiotics (17, 39, 66, 103). Subinhibitory concentrations of certain antibiotics induce the RpoS regulon, because increased energy production and translation capacity in treated cells accelerates nutrient depletion and triggers the stringent response in *E. coli* (103); the RpoS regulon can also be induced by treatment-triggered ROS production (39). Induction of the RpoS regulon increases β-lactam-induced mutagenesis in *E. coli*, *P. aeruginosa*, and *V. cholerae* (66). In these species, mutations are generated by the *dinB*-encoded TLS polymerase, which is regulated by RpoS in addition to LexA (53). These mutations are fixed, however, due to the reduced mismatch repair (MMR) activity mediated by SdsR, the RpoS-controlled small RNA (66).

ANTIBIOTIC-INDUCED RECOMBINATION

Recombination is thought to be under strong selective pressure in bacterial populations, as indicated by the differences in recombination rates between bacterial species (44), as well as between commensal and pathogenic strains of the same species (135). Recombination thus seems to play a crucial role in bacterial evolution. Antibiotics can also affect recombination. Subinhibitory ciprofloxacin concentrations stimulate both intra- and interchromosomal recombination between identical and divergent sequences in *E. coli*, independently of SOS response induction (97). This antibiotic also stimulates recombination in MMR-deficient mutator strains; fortunately, stimulation of homologous recombination appears to be specific to fluoroquinolones, as it was not observed for ten other antibiotics of different chemical classes with distinct molecular targets (97).

A particular case is site-specific recombination, a process by which specialized recombinases recognize and target specific DNA sequences. For instance, the integron integrases are induced by antibiotics in a SOS-dependent manner, in both laboratory (64) and clinical settings (72). The site-specific recombinase gene *ccrC1* is similarly induced by several clinically relevant antibiotics in *S. aureus*, leading to transfer of the SCC*mec* chromosomal cassette, which confers methicillin resistance (94). Similar examples of SOS-controlled, site-specific recombinases and other mobile genetic elements are abundant and have been reviewed recently (49).

ANTIBIOTICS AS SELECTORS OF HYPERMUTABLE BACTERIA

Bacterial cells with increased frequency of mutations (hypermutators or mutators) have an increased probability of acquiring favorable mutations, including those that confer antibiotic

resistance. In certain conditions, hypermutable alleles can thus accelerate the evolutionary rate and confer an indirect selective advantage on the strains that carry them (34, 55). Unfortunately, the opposite is also true, as antibiotic pressure selects for hypermutator clones (102). Selection for a single mutation increases the proportion of mutators in the population from the original 0.001% (the normal frequency in an *E. coli* population) to as much as 0.5%. Successive selection steps can further increase the proportion of mutator strains up to 100% (102). Mutator alleles remain linked to the beneficial mutations they produce, a process known as second-order selection or genetic hitchhiking (102, 142). Because bacterial populations can face successive or alternative antibiotic challenges, particularly in chronic infections, mutators become more frequent in the challenged population. A given antibiotic can therefore select not only for resistance to itself but also, indirectly, for increased capacity to acquire resistance to unrelated antibiotics. This relationship was illustrated by the demonstration that *P. aeruginosa* hypermutable strains are selected in patients with cystic fibrosis or chronic obstructive pulmonary disease; these strains become highly resistant to a large number of antibiotics (100, 120).

Heritable hypermutation in bacteria is mainly due to alterations in genes of the MMR system (*mutS*, *mutL*, *uvrD*), which corrects mismatches produced during DNA replication (119, 120, 131). Deficiency in other antimutator genes, such as *mutT* (which removes 8-oxo-G and 8-oxo-dG) and *mutY* and *mutM* (which eliminate mistakes produced by 8-oxo-dG incorporation into DNA), is also found among *P. aeruginosa* mutator strains in cystic fibrosis patients (29). A noncanonical MMR system was recently identified in the major human pathogen *Mycobacterium tuberculosis*, a species that lacks the classical MutS/MutL protein–based MMR (27). Polymorphisms that affect the activity of NucS, the key protein in this noncanonical MMR, were found in clinical strains; these polymorphisms produce hypermutable phenotypes, which suggests that hypermutators can also be selected in *M. tuberculosis*.

It is argued that the probability of fixation of a resistance mutation is not greatly limited by the mutation supply rate, because bacterial population size in an infected individual exceeds 10^{10}, which implies that preexisting resistant mutants are present in the infecting population when treatment begins (76). In some situations, a high mutation rate could nonetheless be essential for developing resistance. In *E. coli*, clinical resistance to some antibiotics such as fluoroquinolones requires more than one mutation (117), which dramatically reduces the effective population able to acquire resistance by mutation. Most resistance mutations have a high fitness cost, and additional mutations that compensate this cost must be acquired to allow the infection to thrive. In addition, treatments very often consist of a combination of two or more antibiotics. For example, the frequency of mutants resistant to the combination of ceftazidime plus fosfomycin in *P. aeruginosa* PAO1 is far below 10^{-10}, whereas the frequency is considerably higher (10^{-8}) in a hypermutable *P. aeruginosa* PAO1 *mutS* derivative (136). In all these cases, higher mutation supply rates can make the difference between bacterial survival and death.

Inactivation of the MMR system also produces an increase in recombination frequency between two divergent DNA sequences from the same or different bacterial species (132). The probability of acquiring new functions by both mutation and recombination is thus greatly increased in MMR-deficient strains.

IS ANTIBIOTIC-INDUCED GENETIC VARIATION BIOLOGICALLY AND CLINICALLY RELEVANT?

The increased genetic variability provoked by certain antibiotics would potentially accelerate the rate at which resistance is developed. Most experiments have been carried out in in vitro systems, and the biological and clinical relevance of such mechanisms in promoting

evolvability (that is, the ability of a population to generate adaptive variation) is still debated. It is proposed that the genetic novelty produced by subinhibitory concentrations of antibiotics might be insufficient to increase evolvability if population size were severely reduced (33). This is because evolvability depends crucially both on the rate at which genetic diversity is produced and on population size. Using this rationale, Frenoy & Bonhoeffer (52) measured evolvability as the absolute number of rifampicin-resistant mutants in final populations of antibiotic-treated cultures. Their results suggest that despite increasing mutation rates, bactericidal antibiotics such as norfloxacin might in fact reduce evolvability by greatly reducing effective population size (52). Experimental evolution using a subinhibitory ciprofloxacin concentration showed that the SOS response contributes to bacterial fitness without increasing evolvability in *P. aeruginosa* (150). These studies challenge the view of antibiotic-induced mutagenesis as a driver of bacterial evolvability, although the discrepancies observed could be due to dissimilar experimental designs. In contrast, in vivo experimental evidence suggests that antibiotic-induced mutagenesis is crucial for development of resistance. In a paradigmatic example, Boshoff and colleagues (23) demonstrated that deletion of the SOS-controlled *dnaE2* polymerase in *M. tuberculosis* drastically reduces the appearance of resistant mutants following ciprofloxacin therapy in mice. Cirz et al. (30) showed that preventing LexA cleavage renders *E. coli* unable to evolve resistance either to ciprofloxacin or to rifampicin in a mouse thigh infection model. In accordance with these observations, inhibition of the SOS response in a neutropenic mouse model was recently shown to completely abolish emergence of resistant *E. coli* mutants after ciprofloxacin treatment (3).

Evolvability: the ability of a particular organism to undergo evolutionary variation

Despite this accumulating evidence, it is still difficult to distinguish whether the effects of SOS induction in promoting antibiotic resistance stem from increased survival or increased mutation rates. Additional in vivo studies with appropriate controls are needed to solve this question, especially considering that modulation of bacterial evolvability has been proposed as a feasible therapeutic target (31, 37).

TARGETING EVOLUTION OF ANTIBIOTIC RESISTANCE

It is becoming clear that targeting the mechanisms that underlie the evolution of resistance is a promising strategy for lengthening the shelf life of our antibiotic arsenal (13, 31, 37, 146). The objective of these strategies is not to kill bacteria but to inhibit the development and spread of resistance. As resistance to antibiotics depends on generation of genetic variability and the effect of selective pressures, several strategies have been proposed to limit selection of resistant bacteria, such as rational design of treatments to exploit collateral sensitivity networks (77, 85, 122). Here we focus on strategies that target the generation of genetic variability.

SOS Inhibitors

The SOS response is arguably one of the most appealing targets with which to combat the evolution of resistance, at least for two reasons. First, the SOS response is highly conserved in most bacterial phylogenetic groups (48), potentially enabling the use of broad-spectrum drugs. Second, inhibition of the SOS response not only reduces antibiotic-induced mutation, recombination, and HGT rates but also renders bacteria more sensitive to certain antibiotics; in some cases, this leads to complete reversion of bacterial resistance (111, 133). SOS inhibition might also lead to a reduction in pathogenic processes such as persistence, tolerance, infection, and expression of toxins or virulence factors (21, 46, 60, 74). It is therefore not surprising that a multitude of studies have explored SOS inhibition as a therapeutic adjuvant to limit development of antibiotic resistance (**Figure 2**; a comprehensive list is shown in **Table 1**).

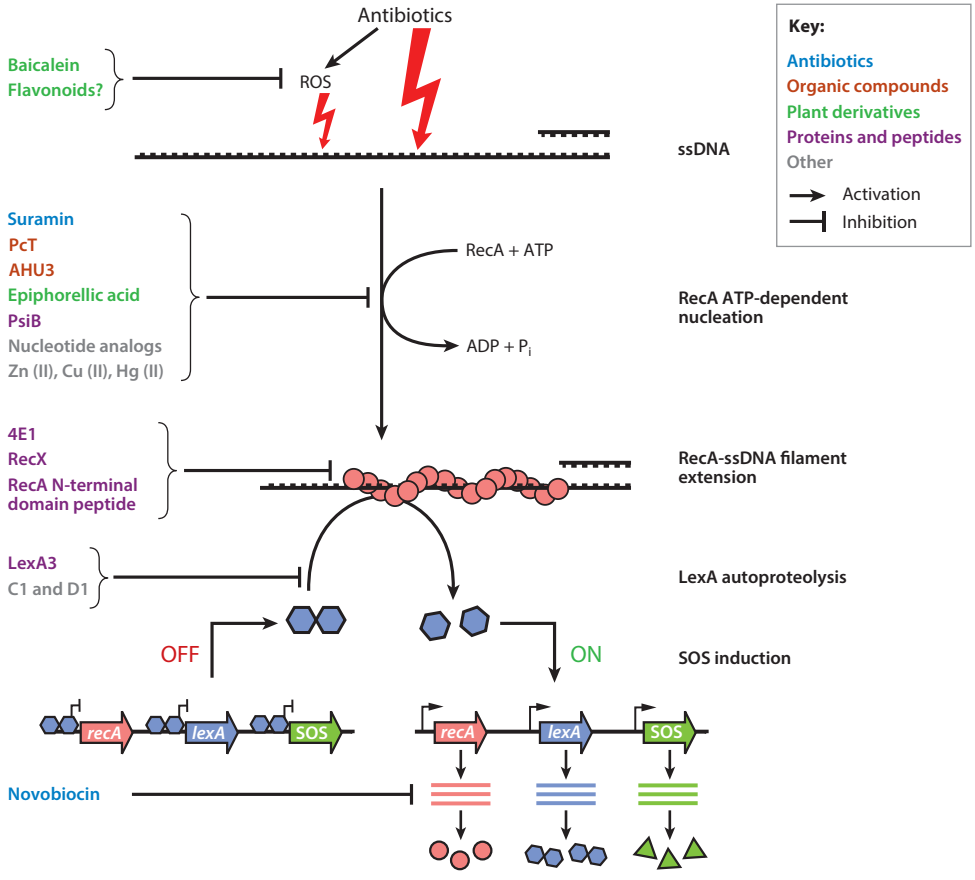

Figure 2

How to suppress SOS-mediated, antibiotic-induced genetic variation. Certain antibiotics cause DNA damage (*red lightning bolts*), either directly or accompanied by ROS induction. DNA damage in turn gives rise to ssDNA, triggering the ATP-dependent filamentation of RecA (*pink circles*). The active RecA-ssDNA filament catalyzes autoproteolysis of LexA (*blue hexagons*), inducing expression of the SOS genes, including *recA* and *lexA*. Several compounds that inhibit SOS induction at different steps are color coded according to class (see key in figure). For detailed information about the compounds with SOS inhibition activity, see the main text and **Table 1**. Abbreviations: PcT, phthalo-cyanine tetrasulfonates; ssDNA, single-stranded DNA.

Some of these approaches have exploited the natural ability of specific bacterial proteins to inhibit the SOS response. For instance, Lu and Collins (99) inserted a gene encoding an uncleavable variant of the LexA protein (*lexA3*) into the bacteriophage M13 genome. Infection of *E. coli* cells with the phage inhibited SOS induction, and the engineered phage potentiated norfloxacin activity in a murine infection model. In a very interesting approach, Yakimov et al. reproduced the RecX mechanism of action (that is, inhibition of RecA filamentation) by designing and synthesizing a small α-helical peptide able not only to inhibit RecA activities in vitro but also to suppress the bacterial SOS response in *E. coli* (159). This opens up new approaches to exploiting the natural activity of some bacterial proteins and peptides, such as RdgC or PsiB, to inhibit RecA-mediated induction of the SOS response (35). Moreover, peptides could be designed to block the activity of specific SOS components such as the $UmuD'_2C$ polymerase (pol V), owing to its precise posttranslational regulation (61).

Table 1 Inhibitors of the SOS response

SOS inhibitor	Bacterial species[a]	Times decrease in mutagenesis (inducer)[b]	Other phenotypes	Proposed mechanism of action	Ref.
Antimicrobials					
Novobiocin	*Staphylococcus aureus*	<3 (CIP)	■ Inhibition of *recA* and *umuCD* expression ■ Inhibition of formation of hemolytic variants and phage transduction	Alteration of supercoiling by GyrB blockage reduces *recA* expression	140, 141
	Acinetobacter baumannii	>100 (UV) >4 (CIP) >2 (TET)	Inhibition of *umuC*, *umuD*, *umuCab* expression after DNA damage	Reduction in the generation of ssDNA regions and in the number of DNA strand breaks made during the excision repair process	80
Amikacin	*Pseudomonas aeruginosa*	~2 (CIP)	Twofold reduction in *recA* expression	ND	152
Suramin	In vitro	ND	Inhibition of ATPase and DNA-strand-exchange activities of RecA	Direct interaction with RecA protein	157
	Mycobacterium tuberculosis	ND	■ Inhibition of ciprofloxacin-induced *recA* gene expression and the SOS response ■ Increase in the bactericidal action of ciprofloxacin	Direct interaction with RecA protein	114
Phage					
Engineered M13-carrying *lexA3*	*Escherichia coli*	ND	Enhanced antibiotic sensitivity (murine model)	Genetic repression of the SOS response	99
Peptides					
4E1 peptide based on RecX	*Escherichia coli*	ND	■ Inhibition of *recA* expression ■ Increased sensitivity to UV	Direct interaction with RecA protein	159
29-Mer peptide based on the RecA N-terminal domain	In vitro	ND	Inhibition of RecA filament assembly	Direct interaction with RecA protein	32
Unclassified					
Compound A1	*Escherichia coli*	ND	Inhibition of the RecA-catalyzed strand-exchange reaction	ND	156
Compounds C1 and D1	*Escherichia coli* and *Pseudomonas aeruginosa* and in vitro	ND	Inhibition of LexA autoproteolysis	ND	110

(*Continued*)

Table 1 (*Continued*)

SOS inhibitor	Bacterial species[a]	Times decrease in mutagenesis (inducer)[b]	Other phenotypes	Proposed mechanism of action	Ref.
Nucleotide analogs					
*N*6-(1-naphthyl)-ADP	In vitro	ND	Competitive inhibition of RecA ATPase activity	Direct interaction with RecA protein	89
Various NTPs	In vitro	ND	Inhibition of ATPase activity of RecA Facilitation of RecA-DNA dissociation	Interaction with DNA-RecA nucleoprotein filament or binding to inactive RecA	155
Metals					
Zn (II)	In vitro	ND	Inhibition of RecA ATPase activity	Metal-dependent aggregation of RecA	90
	Escherichia coli	5–32 (CIP)	■ Inhibition of LexA cleavage ■ Inhibition of *Shiga* toxin production	Perturbation of the RecA conformation such that its ability to bind ATP is reduced	24, 36
Cu (II), Hg (II)	In vitro	ND	Inhibition of RecA ATPase activity	Metal-dependent aggregation of RecA	90
Plant and fungus derivatives					
Epiphorellic acid	In vitro	ND	Noncompetitive inhibition of RecA ATPase activity	Direct interaction with RecA protein	20
Curcumin	*S.* Typhimurium and *Escherichia coli*	2 (UV)	■ Inhibition of UV-induced *umuCD* gene expression ■ Inhibition of phage reactivation	ND	19
	Escherichia coli	ND	Suppression of the SOS response induced by levofloxacin	ND	19
Genistein (flavonoid)	*S.* Typhimurium and *Escherichia coli*	6 (ENU)	Inhibition of *umuCD* gene expression	Reduction of the interaction of RecA protein with ssDNA	107, 161
Daidzein (flavonoid)	*S.* Typhimurium and *Escherichia coli*	ND	Inhibition of *umuCD* gene expression	ND	107
Baicalein (flavonoid)	*Staphylococcus aureus*	4 (CIP)	Inhibition of intracellular ROS and ATP levels	Combined effect of ROS inhibition and inhibition of ATP synthase	123
Tectorigenin (flavonoid)	*S.* Typhimurium	1.7 (AF-2)	Inhibition of *umuCD* gene expression	ND	109
Acacetin, apigenin, luteolin, and quercetin (flavonoids)	*S.* Typhimurium	~2 (AF-2)	Inhibition of *umuCD* gene expression	ND	107

(*Continued*)

Table 1 (*Continued*)

SOS inhibitor	Bacterial species[a]	Times decrease in mutagenesis (inducer)[b]	Other phenotypes	Proposed mechanism of action	Ref.
Phytoncides	*S.* Typhimurium	ND	Inhibition of *umuCD* gene expression	ND	70
Cinnamon oil	*Escherichia coli*	ND	■ Inhibition of Shiga toxin production ■ Decrease in *oxyR*, *soxR*, and *rpoS* expression	ND	143
Ascorbic acid derivative					
Disodium isostearyl 2-O-L-ascorbyl phosphate	*S.* Typhimurium	5 (MMNG)	Inhibition of *umuCD* gene expression	ND	69
Organic compounds					
Phthalo-cyanine tetrasulfonates	*Escherichia coli*	>8 (CIP)	■ Inhibition of RecA ATPase, DNA-binding, DNA-strand-exchange, and LexA-proteolysis activities ■ Potentiation of bactericidal antibiotic killing ■ Inhibition of filamentation and biofilm formation ■ Inhibition of generation of CIP-resistant mutants in a murine model	Direct interaction with RecA protein	3
AHU3 (maleimide)	*Escherichia coli*	ND	■ Inhibition of ATPase activity of RecA ■ Reduction in RecA oligomerization ■ Reduction of MMC-induced phage production Inhibition of *Shiga* toxin production	Covalently binding to one or more of the three cysteine residues present in RecA	74

Abbreviations: AF-2, furilfuramide; CIP, ciprofloxacin; ENU, *N*-ethyl-*N*-nitrosourea; MMC, mitomycin C; MMNG, methylnitronitrosoguanidin; ND, not determined; TET, tetracycline; UV, ultraviolet light.

[a]In vitro refers to experiments performed without bacterial cells (e.g., purified proteins in test tubes).

[b]Compared to the induced control. Data were interpolated from graphs when the actual values were not specified in the text; most values are therefore approximate.

Other potential inhibitors of SOS induction include several especially appealing antimicrobial molecules that have already been approved for human therapy. Novobiocin, an aminocoumarin antibiotic, blocks DNA gyrase and alters DNA supercoiling, which leads in turn to downregulation of *recA* expression in *S. aureus* (140). This downregulation reduces SOS induction and antibiotic-induced mutagenesis in *S. aureus* and *Acinetobacter baumannii* (80, 141). Another example is the antiprotozoal drug suramin, which reduces the SOS response in *M. tuberculosis*, potentiating ciprofloxacin activity (114). The aminoglycoside amikacin was similarly shown to reduce the ciprofloxacin-induced SOS response and mutagenesis in *P. aeruginosa* (152); in other bacterial species, however, aminoglycoside antibiotics were shown to induce the SOS response (10, 11). Antibiotics able to inhibit the SOS response therefore offer a unique opportunity for combined therapies designed both to kill bacteria and to inhibit the evolution of resistance.

Another interesting group of SOS inhibitors stems from plant compounds. Flavonoids such as genistein, daidzein, and baicalein (see **Table 1**) are natural inhibitors of mutagenesis mediated by the SOS response (107, 108, 123, 161). Baicalein can also inhibit antibiotic-induced ROS formation and ATP generation in *S. aureus* (123). Although the common mechanism underlying SOS inhibition by flavonoids is currently unknown, it is tempting to speculate that the antioxidant properties of flavonoids could reduce antibiotic-induced ROS, leading to a reduction in DNA damage (127). The antioxidant properties of other compounds also reduce mutagenesis caused by antibiotics without affecting their bactericidal activity (8).

A number of groups performed high-throughput screenings of chemical compound collections to find molecules able to block SOS induction by targeting specific RecA enzymatic activities. The search for molecules that inhibit RecA ATPase activity has yielded numerous potentially interesting compounds (20, 114, 125, 156, 157), although these SOS inhibitors have only begun to be tested in animal models. Alam and coworkers (3) found that phthalo-cyanine tetrasulfonates (PcT) block the ATPase, DNA-binding, DNA-strand-exchange, and LexA-proteolysis activities of RecA, inhibiting the SOS response. PcT compounds potentiate the bactericidal activity of several antibiotics while reducing the number of mutants after ciprofloxacin treatment, both in vitro and in a murine thigh infection model (3). To the best of our knowledge, this study was the first in which the activity of a SOS inhibitor was demonstrated in vivo, highlighting the potential benefits of SOS inhibition as a therapeutic target and paving the way for future studies.

Despite the therapeutic potential of RecA inhibitors, we should point out that RecA homologs have been found in eukaryotic cells, including human Rad51. RecA inhibitors for therapeutic purposes should thus be designed carefully to selectively target the prokaryotic protein. Conversely, LexA is a potentially safe target, as no eukaryotic homologs have been found. Mo and colleagues (110) developed an academic-industrial partnership to screen 1.8 million compounds for LexA self-cleavage inhibition activity that led to five promising new leads. Inhibition of LexA self-cleavage nevertheless does not increase bacterial susceptibility to antibiotics as much as does inhibition of RecA activity (110), probably due to the high basal RecA and TLS polymerase levels in the noninduced state.

Horizontal Gene Transfer Inhibitors

Inhibiting the mechanisms that underlie the transfer of antibiotic resistance among bacterial pathogens is a strategy that might be used to combat the spread of resistance in some settings. Most work in this area was recently described in various excellent reviews, and we thus highlight only the more innovative and thought-provoking studies.

Inhibition of conjugation is one of the most appealing targets, since many antibiotic resistance genes are associated with large conjugative plasmids. Several molecules have been identified that

show promising conjugation inhibitory activities; unsaturated fatty acids are one of the most efficient. For more information regarding these molecules as well as their mechanism of action, we refer readers to a recent review, Reference 26.

Other studies have focused on directly eliminating plasmids, instead of inhibiting their transfer (4). Plasmid-curing compounds such as acridine orange or ethidium bromide have been known for decades, although most are mutagenic, which would preclude their use in clinical settings. A few recent reports highlight new strategies for plasmid curing. Ojala et al. (118) took advantage of the affinity of the lytic bacteriophage PRD1 for the conjugative pili encoded in a RP4 plasmid and found that the plasmid renders bacteria sensitive to phage killing. By coculturing plasmid-bearing bacteria with the phage, they showed that the plasmid was rapidly eliminated from bacterial populations, even in the presence of antibiotics against which the plasmid provided resistance. Kamruzzaman et al. (83) engineered interference plasmids by specifically deleting toxin genes from toxin-antitoxin systems and antibiotic-resistance genes from clinically relevant plasmids. These attenuated plasmids were able to displace their natural cognates in bacterial populations, both in culture media and in a mouse gut model, revealing a plausible strategy to eliminate prevalent plasmids from bacterial populations (83). For more data on plasmid-curing strategies, we refer readers to a recent review, Reference 91.

CONCLUDING REMARKS

We must understand that bacterial resistance is the unavoidable consequence of the adaptive process of life. To date, no antimicrobial agent has escaped bacterial resistance. Looking at our recent history, resistance is thus probably impossible to eliminate, but we can try to hinder its progress in a cooperative endeavor by different disciplines, including medicine, genetics, microbiology, epidemiology, and sociology, always in the perspective of evolution. There are no simple solutions to this complex problem. Combined interventions are needed against emergence of antibiotic resistance, invasion, and occupation of a niche by resistant clones (14).

This article reviews how antibiotics affect emergence of resistance and the strategies that have been developed to avoid or reduce it, as well as its transmission by horizontal transfer. Most mechanisms reviewed here that promote the evolution of antimicrobial resistance stem from complex metabolic and genetic side effects of antibiotics; they are therefore to some extent the inevitable result of antibiotic use. They can probably be diminished or kept in check using tactics developed to combat the evolution of antibiotic resistance. Although some of these strategies show great potential, there is still a long way to go to effectively limit the generation and spread of antibiotic-resistance genes. More research, especially in in vivo models, is needed to ascertain the safety and efficacy of these compounds. Clinical trials might corroborate their effectiveness and stimulate wider use of these compounds in clinical settings. Such innovative therapies might nonetheless require changes in existing regulations, especially those concerning bacteriophages and genetically modified microorganisms.

It is time to adopt novel therapeutic approaches that target the generation and spread of antimicrobial resistance. It is our view that, in the near future, the combination of antibiotics with evolution-targeting drugs will be essential for offsetting the uncanny ability of bacteria to develop antibiotic resistance.

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