Microbial environments confound antibiotic efficacy

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Despite our continued efforts to assert control over pathogens, more and more bacteria are saying "no" to drugs. It is becoming increasingly apparent that microbial environments, influenced by intracellular and extracellular metabolic processes, modulate antibiotic susceptibility in bacteria. A deeper understanding of these environmental processes may prove crucial for the development of new antibacterial therapies.

The development of antibiotics to combat infectious diseases stands as one of the most significant advances in medicine and public health. Since the discovery of penicillin in 1929, our mechanistic understanding of antibiotics has centered on the chemical inhibition of essential bacterial proteins. To date, this drug-target model has been a success. It has led to the isolation and subsequent chemical enumeration of additional antimicrobials that inhibit DNA, RNA, protein or cell-wall biosynthesis. However, the efficacy of these antibiotics is continually undermined by the preferential selection of resistant mutants, an unfortunate result of antibiotic use. Consequently, we are constantly breeding new strains of antibiotic-resistant bacteria. And, even in the absence of genetic changes, antibiotic-tolerance mechanisms allow subpopulations of bacteria to withstand repeated treatments with lethal antibiotics, which can lead to persistent and chronic infections.

These antibiotic-evasion tactics are particularly worrisome in light of the declining number of new antimicrobials in the developmental pipeline. The scarcity of new drugs reflects, in part, the limits of our drug-target model[1]. Novel therapies, created on the basis of a deeper understanding of bacterial physiology, are needed to counter these antibiotic-insensitive microbes. As we highlight below, there is a growing appreciation for the roles of microbial environments, metabolic stresses within and metabolic influences between microbes in the strategies bacteria use to defend against antibiotics.

Antibiotics induce metabolic stress

Although the direct targets of most antibiotics are well characterized, less is known about the events that lead to cell death after drug-target binding. Systems-level investigations of these events have implicated the role of intracellular metabolism in antibiotic-mediated cell death. A recent study by Dwyer *et al.* captured and analyzed the genome-wide transcriptional response of *Escherichia coli* following exposure to lethal concentrations of norfloxacin, a quinolone antibiotic[2]. Detailed biochemical studies had previously shown that quinolones inactivate topoisomerases, DNA-bound proteins that control chromosome topology. Double-stranded DNA breaks, coupled with stalled DNA replication machinery at these topoisomerase sites, halt cell division and eventually lead to cell death[3]. However, reconstruction of stress responses using biological network analyses revealed that quinolones also induce cell death by generating reactive oxygen species (ROS)[2]. Specifically, it was found that quinolones promote the generation of superoxide, a byproduct of aerobic respiration that attacks iron-sulfur clusters to release ferrous iron. Ferrous iron, in turn, reacts with hydrogen peroxide via Fenton chemistry to generate hydroxyl radicals, which contribute to the lethal effects of norfloxacin.

Building on these findings, Kohanski *et al.* discovered that different classes of bactericidal antibiotics (quinolones, β-lactams and aminoglycosides), regardless of their primary targets, utilize this oxidative-damage cell-death pathway[4]. Importantly, exposure to each antibiotic induces superoxide-mediated destabilization of iron-sulfur clusters by stimulation of central metabolism, eventually leading to the generation of cytotoxic hydroxyl radicals (**Fig. 1a**). This common mechanism of killing was demonstrated in both Gram-negative and Gram-positive bacteria.

The common oxidative-damage cell-death pathway provides an important framework for the development of new antibacterial therapies. For example, compounds that target bacterial systems that remediate hydroxyl radical damage or increase the production of hydroxyl radicals beyond the cell's detoxification capacity could be powerful adjuvants for existing antibiotics. They may also form the basis for novel—and more effective—antibacterial compounds.

This cell-death pathway also provides insight into how bacteria may evade antibiotics through changes in metabolism. We now have a context for understanding how genetic mutations that seem unrelated to an antibiotic's primary target protect a microbe against antibiotic attack. For example, genetic mutations that alter pathways involved in superoxide response[5], the citric acid cycle[6] or aerobic respiration[7] result in reduced antibiotic susceptibility, and this may be due to attenuation of hydroxyl radical production.

More work is needed to improve our understanding of how ROS are generated and how they modulate antibiotic lethality. For example, some Gram-positive bacteria can generate nitric oxide, another type of ROS. Gusarov *et al.* showed that endogenously produced nitric oxide in bacteria can be cytoprotective against a wide range of antibiotics by alleviating the oxidative stress they induce[8]. Nitric oxide generation, produced by the oxidation of L-arginine to L-citrulline, is thus another mechanism by which the intracellular metabolic environment can alter antibiotic susceptibility, and it may represent a useful pathway to target for antibacterial therapies.

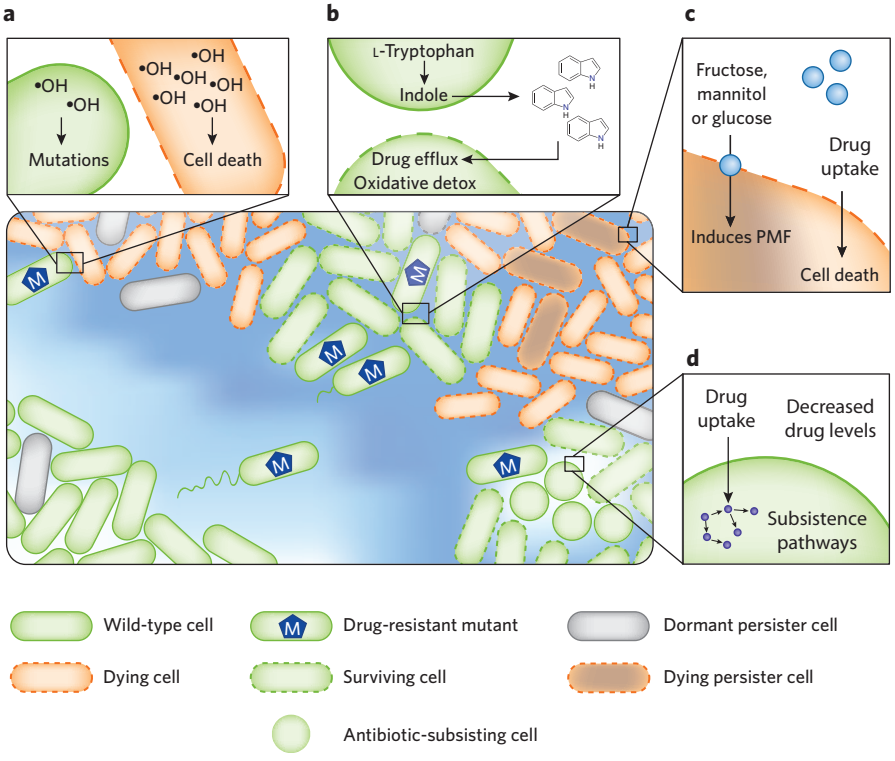

Figure 1 | Microbial environments enable antibiotic resistance and tolerance. A bacterial population subjected to a gradient of antibiotic concentration is shown in the main figure. (**a**) Antibiotics induce formation of deleterious hydroxyl radicals. Under sublethal antibiotic stress, these mutagenic hydroxyl radicals promote the emergence of antibiotic-resistant mutants. Lethal concentrations of antibiotic lead to high levels of hydroxyl radicals, which contributes to antibiotic-mediated cell death. (**b**) An antibiotic-resistant mutant produces indole by catabolizing L-tryptophan. Indole boosts the survival capacity of the population by inducing drug-efflux pumps and oxidative-stress detoxification pathways in the more vulnerable cells. (**c**) A subpopulation of dormant cells, called persisters, is tolerant of antibiotics. Addition of metabolites such as fructose, mannitol or glucose to the extracellular environment generates proton-motive force (PMF), which sensitizes persister cells to aminoglycoside antibiotics. (**d**) Some bacteria are capable of subsisting on antibiotics as a sole carbon source. They may allow a microbial community to evade treatments by reducing the local drug concentration, leading to the formation of antibiotic-resistant mutants via mutagenic hydroxyl radicals, or by eliminating the antibiotic altogether.

A radical source of antibiotic resistance

ROS can also catalyze the development of antibiotic resistance. Hydroxyl radicals, produced as a metabolic byproduct of antibiotic stress, readily damage DNA and thus can be mutagenic (**Fig. 1a**)[9]. A recent study showed that treating *E. coli* with sublethal concentrations of antibiotic induces the formation of hydroxyl radicals, which leads to mutations that enable multidrug resistance[10]. Of note, it was found that this radical-based mechanism can lead to the development of mutant strains that are sensitive to the applied antibiotic but resistant to other antibiotics.

Interestingly, it was also shown that treating a bacterial population with low concentrations of ampicillin, an antibiotic that inhibits cell-wall synthesis, can result in the development of mutants that are resistant to ampicillin as well as kanamycin, a ribosome inhibitor[10]. Multidrug resistance is usually attributed either to mutations that increase drug efflux or to the accumulation of mutations specific to the primary antibiotic targets. However, in this case, the strain that was resistant to ampicillin and kanamycin was found to contain a mutation in a redox sensing system, which is an important part of the common mechanism that influences the regulation of aerobic respiration. This finding indicates that mutations in the common oxidative cell-death pathway can confer multidrug resistance and suggests that deep sequencing studies are needed to identify a broader range of mutations involved in the emergence of antibiotic resistance. This work also suggests that one way to help reduce and contain the spread of new antibiotic-resistant bacteria may be to develop compounds that target ROS-producing systems or error-prone DNA damage repair systems.

Crowdsourcing resistance

Bacteria use a variety of cooperative schemes to survive in diverse conditions[11], including antibiotic stress. In a recent study, we found that bacteria can coordinate the development of antibiotic resistance within a population by sharing indole, a secondary metabolite produced by catabolism of L-tryptophan[12]. Continuously challenging bacterial populations with increasing concentrations of an antibiotic results in the stratification of resistance in the population. The vast majority of isolates in the culture are less resistant than is the population as a whole. In contrast, a small subpopulation of highly resistant cells exists, owing to the accumulation of mutations in antibiotic-specific alleles, and these cells condition the extracellular environment by producing and sharing indole at a fitness cost to themselves. By activating drug pumps and pathways responsible for detoxification of ROS, indole protects the less-resistant cells from antibiotic lethality until they can accrue the mutations that boost innate resistance (**Fig. 1b**). Indole effectively serves as a capacitor for the evolution of antibiotic resistance.

Intriguingly, bis-indoles, compounds based on indole chemistry, have been shown to have antibacterial properties and similar modes of efflux[13]. Lethal indole-like compounds that compete with the uptake and activity of endogenous indole may thus form the basis for powerful antibacterials with reduced rates of resistance.

Bacteria generate a wealth of secondary metabolites that have unknown biological functions. Further investigation may reveal a variety of biogenic molecules in the extracellular environment that modulate antibiotic sensitivity. A recent study by Bernier *et al.* found that gaseous ammonia influences multidrug resistance between physically distant bacterial colonies[14]. It was shown that an *E. coli* colony can generate gaseous ammonia by catabolism of L-aspartate, which increases polyamine levels in physically separated colonies of Gram-positive or Gram-negative bacteria. Increases in intracellular polyamines lead to alterations in membrane permeability to different antibiotics and enhance protection against oxidative stress. These findings indicate that ammonia production and polyamine synthesis may be important pathways to target as part of new therapies.

Together, these studies suggest that efforts to combat antibiotic resistance may be complicated by bacterial signaling mechanisms and cooperative survival strategies. Thus, a better understanding of how bacteria work together to survive stressful, unpredictable environments may

prove crucial for the design of effective clinical interventions.

Engineering reduced antibiotic tolerance

Persisters are dormant bacteria that can withstand, without genetic changes, lethal concentrations of antibiotics. These tolerant subpopulations have been implicated as driving forces behind chronic and recurrent infections[15–17]. Diverse processes and conditions contribute to persister formation, and antibiotic tolerance is achieved, in part, by the induction of pathways that promote cellular quiescence[18]. It was recently shown that metabolic stimulation can sensitize bacterial persisters to antibiotic treatment. Specifically, Allison *et al.* found that addition of specific metabolites (such as fructose, mannitol or glucose) to the extracellular environment could potentiate the activity of aminoglycosides, antibiotics that target the ribosome, in Gram-positive and Gram-negative persisters[19]. These metabolites induce the generation of proton-motive force, which is required for the uptake of aminoglycosides (**Fig. 1c**). This work showed that bacterial persisters, although dormant, are primed for metabolite uptake, central metabolism and respiration. Importantly, the effectiveness of this metabolite-based strategy was validated in a mouse urinary tract infection, demonstrating that the approach can be used in complex, *in vivo* environments.

The use of extracellular metabolite dosing to provoke diverse energetic pathways may enable the use of other antibiotics in eradicating persisters. Intriguingly, a recent chemical screening effort to find compounds that would awaken persisters identified a novel molecule that sensitizes persisters to antibiotics by selectively inducing their growth[20]. The specific mechanism by which this compound induces the reversion of persisters to antibiotic-sensitive cells is unknown at present. Further investigation of the pathways activated by this compound and more extensive study of the basic physiology of bacterial persisters may provide important insights that could be harnessed to engineer extracellular environments to reduce or eliminate antibiotic tolerance.

Ecological niches

Our understanding of how microbial environments contribute to antibiotic resistance and tolerance remains incomplete. Diverse species of bacteria coexist in nature and are subject to a variety of physical constraints. Deconstructing natural bacterial communities may help to reveal how diverse bacteria modulate their extracellular environments to enable antibiotic resistance and tolerance. For example, Dantas *et al.* recently isolated, from natural soil samples, bacteria that can subsist on antibiotics as a sole carbon source[21]. Surprisingly, bacterial subsistence pathways exist for many antibiotics, both natural and synthetic. It is intriguing to consider that, by devouring antibiotics and thereby removing them from the environment, these isolates may serve to alleviate antibiotic stress on a bacterial community (**Fig. 1d**).

Although antibiotic catabolism has not yet been identified in human pathogens, it may be an unrecognized mechanism that promotes the antibiotic resistance and tolerance found in recurrent polymicrobial infections[22,23]. Identification of additional subsistence pathways and the conditions that lead to their activation will be required to ascertain their pertinence to the development of problematic infections.

Natural niches are composed of complex microenvironments that bacteria can use to enhance their survival capacity. Simulation of these natural niches may reveal the temporal dynamics by which antibiotic resistance occurs. In a recent study, Zhang *et al.* found that microenvironments accelerate the emergence and fixation of antibiotic-resistant mutants[24]. A microfluidic device, consisting of a simplified network of interconnected microenvironments, was developed to mimic the metabolic gradients that bacteria naturally encounter. Resistant mutants, which emerge as rapidly as 10 hours after antibiotic treatment, preferentially move to regions that have large gradients of antibiotic stress. These regions confer a growth advantage to the resistant strains, which subsequently outgrow their antibiotic-sensitive progenitors to dominate the bacterial population. This phenomenon, which cannot be observed using conventional laboratory techniques, illustrates the value of developing new methodologies that mimic natural bacterial environments.

Summary

The increasing prevalence of bacteria that are insensitive to our current antibiotics emphasizes the need for new antimicrobial therapies. Conventional approaches to antibacterial development that are based on the inhibition of essential processes seem to have reached the point of diminishing returns. The discovery that diverse antibiotics stimulate a common oxidative cell-death pathway represents a fundamental shift in our understanding of bactericidal antibiotic modes of action. A number of studies, as discussed above, also provide hints about how intra- and extracellular metabolism can enable antibiotic resistance and tolerance (see **Table 1**).

We have, nonetheless, just begun to understand the repertoire of tactics that bacteria use to evade antibiotics. Biosynthetic pathways for natural antibiotics are ancient, and numerous mechanisms for antibiotic resistance and tolerance are likely to have evolved over the past few million years[25]. Unraveling these mechanisms will require concerted efforts by chemical biologists, microbiologists and clinicians. These efforts will benefit from the use of metabolic models and

Table 1 | Chemicals in microbial environments that affect antibiotic resistance or tolerance

Biomolecules	Source	Activity
Reactive oxygen species		
Superoxide	Aerobic respiration	Leaches ferrous iron from iron-sulfur clusters
Hydroxyl radicals	Fenton reaction	Is mutagenic at low levels and lethal at high levels, causing damage to DNA, lipids and proteins
Nitric oxide	L-Arginine	Reduces hydroxyl radical concentrations by suppressing the Fenton reaction and activating catalase, an antioxidant enzyme
Biogenic molecules		
Indole	L -Tryptophan	Induces drug-efflux pumps and oxidative stress–protection pathways
Gaseous ammonia	L -Aspartate	Modifies membrane permeability and increases resistance to oxidative stress
Carbon sources		
Mannitol, glucose, pyruvate, fructose	Supplied	Stimulates generation of proton-motive force in persisters for uptake of aminoglycosides, antibiotics that target ribosomes

other network-biology approaches to guide investigation of processes that modulate antibiotic susceptibility. Importantly, by helping to identify common points of vulnerability as well as key differences between pathogens, these models may lead to the development of effective adjuvants, novel antibiotics and new antimicrobial strategies.

There is also a crucial need to better understand how bacteria within a population cooperate to overcome antibiotic treatments. Such investigations may benefit from the use of novel chemical probes and experimental techniques to interrogate the physiology and functional dynamics of natural microbial communities. Insights gained from these studies will augment metagenomic models that can be used to identify biomolecules responsible for these cooperative strategies. Leveraging chemical biology methodologies and systems-biology approaches for further studies of microbial environments may reveal a wealth of untapped targets for the development of novel compounds to counter the growing threat of resistant and tolerant bacterial infections. ■

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Competing financial interests

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