

The Fast Track to Multidrug Resistance

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In this issue of *Molecular Cell*, Kohanski et al. (2010) demonstrate that even subinhibitory concentrations of bactericidal antibiotics result in the generation of reactive oxygen species, leading to an increase in mutation rate and the emergence of multidrug-resistant bacterial strains.

Bacteria employ elaborate systems to avoid accumulating mutations in their DNA (Kunkel and Erie, 2005), which are most often disadvantageous or lethal. Nevertheless, during times of environmental stress, such as the introduction of an antibiotic, mutations can be beneficial and even protect cells from death. Under these circumstances, bacteria may benefit from increasing their baseline mutation rate (Chopra et al., 2003).

Building on earlier work, Jim Collins and his team found that prolonged exposure to weakly inhibitory concentrations of antibiotic can springboard *Escherichia coli* and *Staphylococcus aureus* from drug-sensitive to multiply drug-resistant (MDR) (Kohanski et al., 2010). The treatment accomplishes this feat by boosting the bacteria's mutation rate many-fold, increasing the population's overall sequence diversity, and enriching it for potential drug-resistant mutants.

Bacteria treated in this way fared much better than untreated bacteria when challenged with a variety of drugs (Figures 1A–1D), producing mutants of all types at higher frequencies than untreated cells (Figures 1G and 1J). For example, treatment with sublethal levels of ampicillin, a β -lactam, increased the number of resistant mutants to a completely different class of antibiotic, the fluoroquinolones. In fact, the great majority of mutants that arose showed additional cross-resistance to drugs they had never encountered (Figures 1E and 1F), possibly an additional consequence of the increased mutagenesis. Many of these mutants remained fully sensitive to ampicillin, suggesting that low concentrations of drug appear to stimulate random mutagenesis despite applying no overt selective pressure.

In contrast, mutants that appeared after strong selection by high concentrations of drug without prior exposure to low levels of drug demonstrated little cross-resistance to other classes of antibiotics. For example, primary selection with ampicillin elicited only ampicillin mutants in these experiments, none of which grew when replica-plated onto other drugs (Figures 1K and 1L). Similarly, mutants that underwent primary selection with norfloxacin grew only on norfloxacin and no other class of drug, a consequence of simultaneous mutagenesis and selection.

Previously, the authors identified the production of reactive oxygen species (ROS) as a common step in antibiotic-mediated cell death (Kohanski et al., 2007). Here, they propose that even weak antibiotic exposure boosts intracellular ROS production. These toxic ROS batter the cell, damaging proteins, lipids, and DNA. Indeed, the more ROS a cell population produced, the more rapidly they were found to mutate. Suppressing ROS with quenchers like thiourea or subjecting cells to anaerobic conditions reversed the effect, returning rates to their untreated levels.

An elevated mutation rate may derive in part from ROS damaging DNA directly. However, DNA damage has its own downstream effects. These include the activation of the bacterial SOS DNA damage response pathway, which upregulates specialized polymerases able to synthesize DNA across lesions at the cost of fidelity (Ratray and Strathern, 2003), as well as an increase in recombination (Figure 2). It is not known which of these mechanisms dominates under these circumstances or if many contribute.

Many classes of bactericidal antibiotics provoke bacteria to generate ROS

(Kohanski et al., 2007). Consequently, these results carry a startling corollary: that any bactericidal drug in a therapeutic cocktail may assist bacteria in attaining resistance to the entire combination. In other words, antibiotics may behave functionally as mutagens, especially at low concentrations.

The ramifications of these results are broad because high concentrations are difficult to achieve and maintain in most environments. Antibiotics routinely fall to subinhibitory levels in human patients between doses or in drug-inaccessible tissues. Animal feedstock frequently possesses low levels of antimicrobials as well, as do natural environments like soils. As bacteria move among these environments, mutation rates may be considerably higher than in vitro estimates would predict.

In niches demanding rapid adaptation, higher mutation rates can provide a selective advantage. Classically, bacteria achieve these higher rates by disrupting the DNA mismatch repair (MMR) system either genetically (Giraud et al., 2001) or via regulation (Saint-Ruf and Matic, 2006). The production of ROS provides an alternative pathway bacteria might exploit to modulate their mutational response that is independent of MMR and that uses antibiotics as a signal.

In this study, the authors identified an interesting mutation in the *acrAB* gene (Kohanski et al., 2010), previously found to be involved in ROS-mediated cell death (Kohanski et al., 2007), which may bestow simultaneous resistance to numerous drugs. However, achieving MDR status typically requires multiple independent chromosomal modifications. MDR strains of *Mycobacterium tuberculosis*, for example, have become so through a series

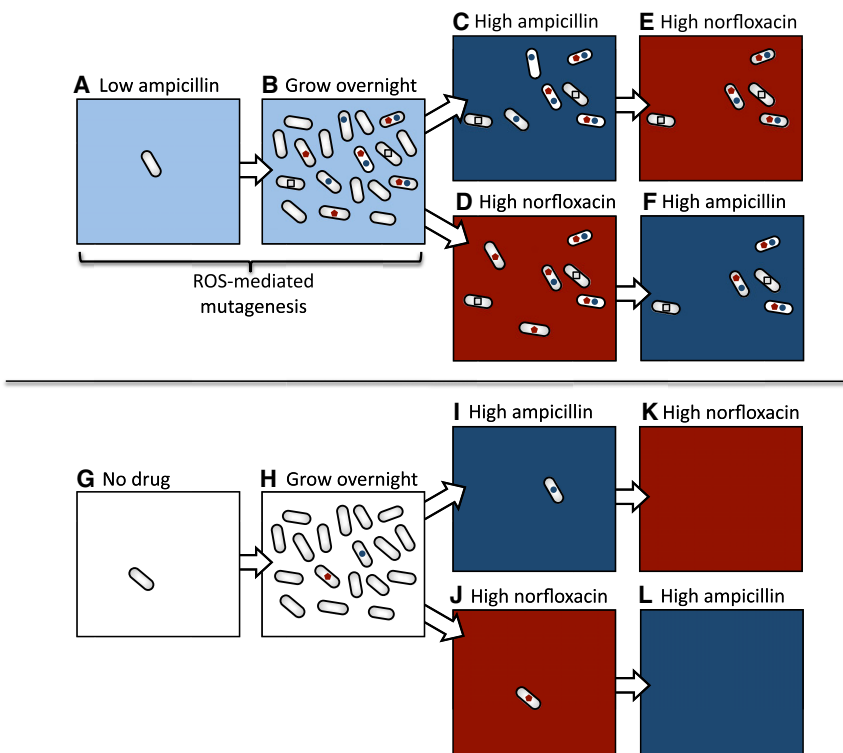


Figure 1. Sublethal Quantities of Bactericidal Drugs Promote Generation of Mutants
(A–L) Panels describe the flow of time as a population of drug-sensitive bacteria (white rods) are exposed to ampicillin or norfloxacin (blue and red background colors, respectively). Mutations conferring ampicillin and norfloxacin resistance are depicted as blue circles and red hexagons, respectively. Single mutations conferring simultaneous resistance to both drugs, such as *acrAB*, are depicted as yellow squares. Bacteria treated with sublethal levels of ampicillin (light blue) exhibit elevated mutation rates (A and B), yielding an increase in the number of high-level norfloxacin (C) and ampicillin (D) mutants. Resistant cells generated in this way show a high likelihood of demonstrating cross-resistance to other heterologous drugs (E and F). Lacking ampicillin pretreatment, a low basal level of mutation occurs (G and H), producing few high-level norfloxacin (I) and ampicillin (J) mutants and an undetectable level of bacteria resistant to both (K and L).

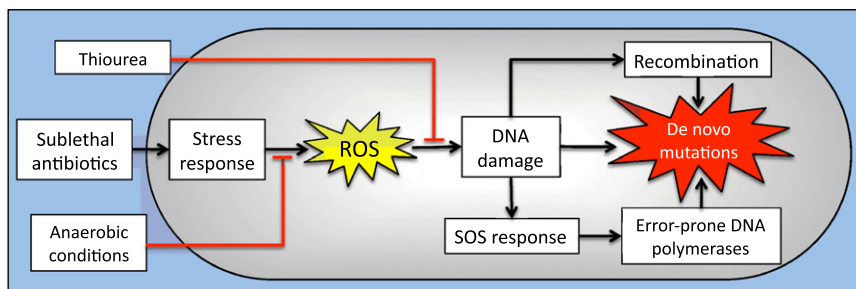


Figure 2. Key Elements in the Pathway Leading from Drug Exposure to Increased Mutation Rate
Antibiotics indirectly cause bacteria to generate ROS via a stress response (Kohanski et al., 2007), which leads to de novo mutations directly, via the SOS response, and through recombination. Thiourea quench ROS, and anaerobic conditions prevent their formation, blocking this mutagenesis.

of chromosomal changes (Banerjee et al., 2008).

Even allowing for a 10-fold increased mutation rate, the acquisition of more than one resistance marker in conditions

that do not directly select for them would still appear to be a rare event. Nevertheless, one isolated mutant in this study, when examined by PCR, possessed at least two resistance-conferring SNPs:

one in the promoter of the efflux pump *acrAB*, the other in DNA gyrase subunit *gyrA*, which quinolones target. This observation suggests that multidrug resistance can be achieved in relatively small populations of cells and in the absence of a step-by-step series of purifying selections, such as a series of failed monotherapy attempts. Instead, factors other than their formation may be the limiting hurdles to MDR bacteria's broader emergence, such as a lower initial fitness when they do appear (Lenski, 1998).

Single-cell microscopy, a technique rapidly expanding in power and throughput (Locke and Elowitz, 2009), may help clarify the role ROS play in stimulating mutation. Because ROS positively correlate with mutation rate on the population level (Kohanski et al., 2010) and significant cell-to-cell variation exists in the level of ROS (Kohanski et al., 2007), the rate of mutagenesis might reasonably vary from cell to cell as well. Cell populations could exploit such heterogeneity, allowing some cells to mutate rapidly while others remain intact.

These results remind us how little we know about how resistance is generated at the molecular level and underscore why preventing the emergence of these lethal strains remains such a formidable challenge.

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