Antibiotic Resistance, Not Shaken or Stirred

Microdevices for microbial culture may better capture the evolution of antibiotic resistance in real-world environments.

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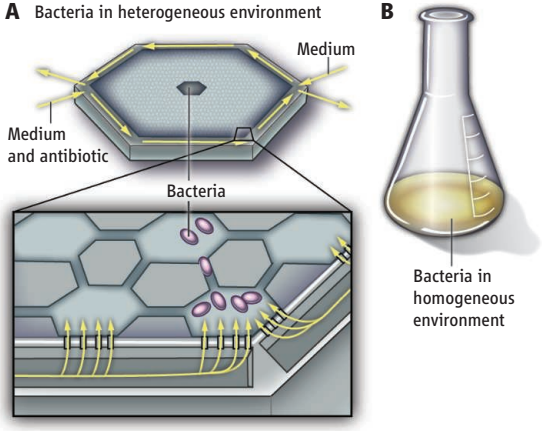
James Bond preferred vodka martinis shaken, not stirred, displaying impressive discriminatory power. Bacteria may be similarly discerning. Zhang *et al*. (*1*) abandoned the standard lab practice of growing bacteria in shaking homogeneous liquid cultures in favor of fabricated microenvironments and report, on page 1764 of this issue, that bacteria can tell the difference. They evolve antibiotic resistance far more rapidly in the structured environment. Evolution seems to work differently in the microenvironment, and perhaps more like evolution in the real world.

In the human body, bacteria encounter heterogeneous environments full of transient chemical and nutrient gradients. Antibiotic gradients can arise when a patient begins and ends therapies, or forgets doses. They might form across spatial heterogeneities as well. For example, the concentration of an antibiotic may be high in blood but low in less permeable dense bacterial clumps or biofilms. To simulate real-world conditions, Zhang *et al*. built a microfluidic device of tiny chambers to create gradients of both a specific chemical and nutrients. The authors then assessed the effect of the microenvironments generated within these chambers on bacterial populations grown in them. This approach may more accurately reflect encounters of microorganisms with chemicals and nutrients in the heterogeneous range of niches they encounter in the real world, such as in soil or within an animal's body. They found that when bacteria (*Escherichia coli*) are grown in a heterogeneous environment that includes a steep concentration gradient of the antibiotic ciprofloxacin (cipro), they show surprisingly rapid and repeatable acquisition and fixation of cipro-resistance mutations compared with bacteria in homogeneous environments.



Microenvironment for bacterial evolution. Fabricated microenvironments, such as the microfluidic device described by Zhang *et al.* (**A**), may provide a relevant approach to understanding microbial evolution in real-world environments that are spatially complex and full of chemical and nutrient gradients, and so differ from homogeneous liquid cultures (**B**).

The microenvironments devised by Zhang *et al*. are chambers within a device (1200 hexagonal wells etched in a silicon wafer) that are interconnected by channels and imbued with nutrient medium through nanoslits (see the figure). Medium flows into the array from two sides of the device: one side with and the other without cipro, thus creating an antibiotic gradient from bottom to top of the device. Inoculated in the center of the device, the bacteria deplete the nutrients locally and then move toward the nutrient-rich periphery through the channels and chambers. Bacteria grow at the periphery of the device but only where the cipro concentration falls below inhibitory levels.

Under these conditions, the steepest point in the cipro gradient is at the periphery where there is a convergence of flow between medium that contains and lacks cipro. The authors found this to be a "Goldilocks point"—a spot at which conditions are just right for de novo cipro-resistant mutants to become fixed in the population. This occurred repeatably, reflected by the accumulation of fluorescent-labeled cells at this point. The increased fitness of these antibiotic-adapted cells allows their growth in unoccupied niches with high cipro concentrations. In addition, the adaptation to cipro occurred rapidly (10 hours with an initial

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inoculum of 10^6 cells). Decreasing the initial inoculum to as few as 100 cells increased the time to adaptation at the Goldilocks points, but did not eliminate the phenomenon, indicating that de novo mutations underlie the increased antibiotic resistance. Perhaps most surprisingly, whole-genome sequencing of three independent adapted isolates revealed the same four mutations fixed in each—single base changes in genes encoding a bacterial gyrase, a component of a transporter of the sugar ribose and some antibiotics, and a negative regulator of drug efflux pumps. By their identity and by the known functions in resistance and sensitivity of the genes bearing the mutations, each mutation appears to play a functional role in the resistance, suggesting extremely rapid fixation of multiple mutations conferring the adapted phenotype. The order in which these mutations occurred or were fixed is not known.

What is special about the Goldilocks point could be both the ecological niche, where resistant mutants succeed, free from competition with their antibiotic-sensitive parents, and also the steep concentration gradient of the stressor cipro, which may favor stress-induced mutagenesis (*2*) by the antibiotic (*3*). Though stress-induced increase of mutation rate can be correlated with increased (though still rare) coincident nonadaptive mutations (*4*), this was not seen by Zhang *et al*. in the small sample examined, either because it was rare (as expected) or possibly because of selection for mutants without additional potentially deleterious mutations. An intriguing question is whether error-prone DNA polymerases, stress-response regulators, and other hallmark proteins and pathways of stress-induced mutagenesis (*2*), including to cipro resistance (*3*), are required for rapid evolution in the nanoenvironments. Such requirements would be expected if stress-induced cipro-resistance mutagenesis underlies the de novo rapid mutagenesis. Some of the proteins required for stress-induced mutagenesis are required for adaptation to long-term liquid culture (*5*).

Zhang *et al*. also noted that control experiments with bacteria placed into liquid shaking (homogeneous) microtiter wells produced no resistant mutants, nor did seeding 10^8 cells onto agar Petri plates with a cipro gradient. Both findings imply the need for the structured environment in which steep gradients facilitate the rapid evolution of resistance by multiple mutations. This fast fixation in the microenvironment confirms predictions from recent modeling (*6*), and the modeling of Zhang *et al*., in which heterogeneous environments speed evolution.

The rapid, repeatable acquisition and fixation of four adaptive mutations appears to differ from results obtained on evolution of shaking liquid cultures serially transferred for thousands of generations (*7*). A hallmark of experimental evolution under serial transfer conditions is that the first adaptation often leads to the greatest increase in fitness, and subsequent adaptations are fixed slowly and contribute decreasingly to the organism's fitness in the evolving environment (*7*, *8*). Thousands of generations appear to occur before adaptation (*7*, *8*). The results from homogeneous liquid seem to differ from those in the structured environment. The rapid acquisition and rise of adaptive mutants in the nanoenvironments more closely resembles cells in continuous liquid growth under strong increasing selection (*9*) than long-term serial flask transfer.

Supposing that some parts of the human body resemble heterogeneous microenvironments more than they do Petri plates and flasks, the structured environments of Zhang *et al*. would appear to provide an intuitively more relevant model for the evolution of antibiotic resistance, in at least some real-world situations. More than that, their results indicate that the differences between homogeneous and heterogeneous environments matter for outcomes. Structured environments may provide more realistic experimental platforms for determining molecular and ecological mechanisms of antibiotic resistance in clinical settings where bacteria are not shaken or stirred.

References

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CHEMISTRY

Ironing Out Hydrogen Storage

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The use of formic acid for storage of hydrogen fuel is enabled by an iron compound that catalyzes dehydrogenation.

The large-scale use of hydrogen (H_2) as a clean transportation fuel—one of the great ambitions in the "hydrogen economy" (*1*)—must overcome several engineering issues. The flammability of H_2 presents a safety hazard, and storing it under pressure, even with absorbent materials, adds unwanted weight to vehicles. One solution to this storage problem is to "carry" H_2 in more stable chemical compounds that have a high hydrogen content. Ideally, these compounds are liquids at room temperature for easy delivery and release H_2 with only a small release of heat to avoid energy losses. Small organic molecules such as alcohols (for example, methanol) or formic acid (HCOOH) fulfill these criteria. The release of H_2 (and CO_2 as a by-product) from HCOOH does not proceed spontaneously, and suitable catalysts are required for its dehydrogenation. On page 1733 of this issue, Boddien *et al*. (*2*) report a molecular iron complex that catalyzes H_2 formation from HCOOH both at high rates and for many catalytic cycles.

Despite being a rather good H_2 carrier, use of HCOOH has the problem that many of the catalysts required for its dehydrogenation in solution have been based on noble metals such as rhodium, iridium, ruthenium, or platinum (*3*, *4*) that are scarce and expensive. In nature, H_2 formation and oxidation are catalyzed by hydrogenases (H_2ases), a class of metalloenzymes the active sites of which contain the earth-abundant first-row transition metals iron or nickel (or both) (*5*). Many recent catalyst-design efforts attempted to mimic the structure and function of the active sites of H_2ases (*6*–*9*). However, Boddien *et al*. took a different approach by screening first-row transition metal catalysts for HCOOH dehydrogenation made from many different metal precursors and ligands in a combinatorial manner (*10*).

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