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Antibiotic Resistance Not Shaken or Stirred

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James Bond preferred vodka martinis shaken, not stirred, displaying impressive discriminatory power. Bacteria, it seems, are similarly discerning. On page ??? of this issue of *Science* (1), a new study abandons the standard laboratory practice of shaken or stirred homogeneous bacterial culture in favor of growth in a heterogeneous engineered microenvironment, and it appears that the bacteria can tell the difference. They evolve antibiotic resistance alarmingly rapidly in the structured environment.

Human bodies are heterogeneous, solid and liquid, and are expected to house transient chemical gradients of nutrients and antibiotics. Antibiotic gradients occur at least when patients begin and end therapies, when they forget doses, and might form across spatial heterogeneities, for example high in blood but low in less permeable dense bacterial clumps or biofilms. To simulate real-world conditions, Zhang *et al.* (1) built tiny chambers (Figure) in which to create chemical gradients, to isolate bacterial subpopulations spatially, and then observe evolution of resistance to the antibiotic ciprofloxacin (cipro). The authors found surprisingly rapid and repeatable acquisition and fixation of resistance mutations in their experimental populations of *Escherichia coli*.

Their microenvironments contain chambers interconnected by channels and imbued with growth medium through nanoslits (Figure C,D). Medium flows into the array from both sides, one side with and the other without cipro (Figure A), creating a cipro gradient from “bottom” to “top” of the device (Figure B). Simultaneously, the bacteria create nutrient gradients. Inoculated in the center of the device, the bacteria move toward the nutrient-rich periphery through the channels and chambers (Figure D). At the periphery they grow, but only where the cipro concentration falls below inhibitory levels (1).

Under these conditions, the authors find, first, that the steepest point in the cipro gradient at the periphery is a “Goldilocks point” (Figure B), where growth is slowed and *de novo* cipro-resistant mutants are fixed in the population. This occurs repeatably, seen as a green pile-up of their GFP-producing cells at this point. The increased fitness of the adapted cells allows growth in unoccupied niches with high cipro levels (1). Second, the adaptation to cipro occurred rapidly (10 hours with an initial inoculum of 10^6 cells). Third, 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, fourth, whole-genome sequencing of three independent adapted isolates revealed the same four mutations fixed in each (1). Interestingly, by their identity, and by the known functions in resistance/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 points could be both the ecological niche, where resistant mutants succeed unmolested by competitors, and also the steep stress gradient, which may favor stress-induced mutagenesis (2) induced by ciprofloxacin (3). Though stress-induced turn-up of mutation rate can be correlated with increased (though still rare) coincident non-adaptive mutations (4), this was not seen in the small sample examined (1), 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/pathways of stress-induced mutagenesis (2), including to cipro-resistance (3), are required for rapid evolution in the nano-environments. 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 were required for adaptation to long-term liquid culture (5).

Control experiments with bacteria placed into liquid shaking (homogenous) microtiter wells produced no resistant mutants (1). Nor did seeding 10^8 cells onto agar Petri plates with a cipro gradient, both implying the need for the structured environment in which steep gradients facilitate the rapid evolution of resistance by multiple mutations (1). This fast fixation in the microenvironment confirms a prediction from recent modeling (6), as well as the modeling of the current authors (1), in which heterogeneous environments accelerated 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). One of the hallmarks 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 (7-9). Thousands of generations appear to occur prior to adaptation (7, 9, 10). The results from homogenous liquid seem to differ from those in the structured environment. The rapid acquisition and rise of adaptive mutants in the nano-environments more closely resembles cells in continuous liquid growth under strong increasing selection (11) than long-term serial flask transfer.

Supposing that some parts of human bodies 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. Experimental evolution in 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.

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Figure. Microenvironment for experimental evolution of antibiotic resistance

(A) The microfluidic chamber of Zhang *et al.* (1) creates a heterogeneous environment with gradients of antibiotic and nutrients, through which motile bacterial cells migrate. Nutrient medium with the antibiotic cipro flows into the chamber's left side and medium without antibiotic into the right, creating a cipro gradient from bottom (green, panel B) to top. (B) The steepest gradients occur at "Goldilocks points" where, repeatably, cipro-resistant mutants are initially established/fixed in the evolving populations. (C) The large hexagonal microchamber in A comprises 10μm-deep hexagons connected by 200μm-long channels through which the bacteria migrate towards nutrients and away from cipro, a stressor, until spontaneous resistance arises (red cells in panel D). (D) Close-up of a Goldilocks point. Resistant mutant cells move towards nutrients, irrespective of cipro, and can fix in the population at those points, whereas sensitive cells might not, despite their need for the nutrients.