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The Goldilocks Principle and Antibiotic Resistance in Bacteria

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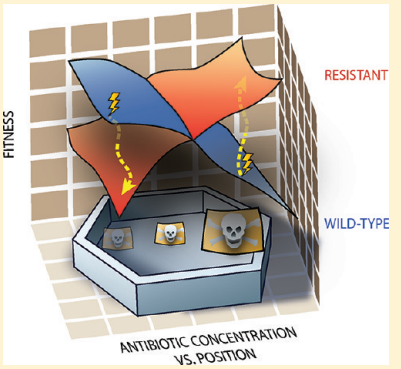
ABSTRACT: We have designed and fabricated a microecology to mimic a naturally occurring bacterial culture, which includes the stress gradient, metapopulation, and cellular motility. In this microecology, we show that it is possible to fix the resistance to the mutagenic antibiotic Ciprofloxacin in wild-type *Escherichia coli* within 10 h. We found the evolution of resistance is further accelerated in microecology if bacteria have already acquired the phenotype of growth advantage at the stationary phase (GASP).

KEYWORDS: antibiotics, resistance, evolution



INTRODUCTION

For those unfamiliar with the old Grimms Brothers fairy tale about Goldilocks and the Three Bears, Goldilocks was a small girl who wandered into a temporarily vacated house owned by a bear family and sampled the three bears' wares for the "just right" combination of taste, fit, and comfort, which envitably ended up being the baby bear's portion.[1] Like Goldilocks' need for the just right parameters, evolution proceeds most rapidly when there is the just right combination of a large number of mutants and rapid fixation of the mutants.[2] "Fixation" means that all of the individuals in that population have a particular mutation. Although evolution occurs in well-stirred chemostats without such Goldilocks conditions,[3] natural environments are rarely well-stirred in nature, as Darwin realized on the Galapagos Islands.[4] For complex environments such as the Galapagos Islands, spatial population density gradients and movement of mutants along these population density gradients can be as important as genomic heterogeneity in setting the speed of evolution.

We follow here in the footsteps of Wright, who emphasized the importance of population sizes.[5] Most evolution experiments are done with the limit of large numbers of individuals, where basically we can expect evolution to move along an adiabatic path on a fitness landscape. If the population number gets small enough, the evolution dynamics can become nonadiabatic and move out of equilibrium, as we graphically describe in Figure 1. We have recently published an article showing that the principles outlined here work in accelerating evolution,[6] we will present here in more detail some of the aspects of how this was done and present new data on how mutant bacteria that are genetically selected to already show growth under stress have even greater acceleration of evolution to antibiotic resistance.

Intuitively, the need for a combination of small populations, population density gradients, and motility of the mutants for rapid evolution of resistance comes from the following considerations: If a mutation occurs which increases the fitness of a mutant, the mutation can be fixed if the mutant moves with velocity $\vec{v}$ down the population gradient into regions of lower population density.[7] This stepwise movement of motile mutant bacteria via successive mutations into regions of higher stress is accelerated if the mutation rate is very high and the population density gradient $-\nabla N$ is very steep. A rare mutation which confers resistance may not be fixable if the local wild-type population is very high, but a resistant mutation in a region of low population can result in rapid fixation. By creating a gradient in nutrients which generates a gradient in population density, the evolution will occur more rapidly not in the high population density regions but rather to the lower population densities, if the organisms are motile.

The phenotypic state of the organism is also a potential controller of the rate of evolution dynamics. If the initial strain of organism is a wild-type one which is not adapted to stress, it is possible that substantial mutations must occur and be fixed before the organism can evolve a true resistance to a highly stressful environment and propagate. However, if the strain has a preexisting genotype which gives rise to a initial resistance to stress, it may require less mutations for full resistance to evolve. In the case of bacteria, which we study here, it has been shown by Kolter and his colleagues that bacteria held in stationary phase over a period of several days evolve, in spite of the fixed number

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of bacterial cells in stationary phase, to a mutant which is adapted to the high stress conditions generated by stationary phase. These mutants, called growth advantage in stationary phase (GASP) mutants, have the ability to divide (slowly) even under high stress conditions. We will test in this paper if GASP mutants can evolve with resistance to genotoxic antibiotics at a faster rate than wild-type bacteria do.

The construct of the wild type and the GASP mutants (growth under stationary phase) *Escherichia coli* with chromosomal green fluorescent protein (GFP) expression has been described in previous work.[8] The GASP mutant has an interesting genetic rearrangement which leads to the GASP phenotype. The main difference from the wild-type alleles is a duplication in the GASP mutant of the rpoS 819 of sequence (gcaggggctgaatatcgaa).[9] We have shown earlier in microhabitat work that these GASP mutants coexist with wild-type bacteria in a synergistic manner[10] and under high stress can grow to high densities. The basic mechanism for this stress adaptation is that the allele confers a strong competitive fitness advantage under basic pH conditions, and during the death phase of stress these GASP mutants are able to adapt using a metabolism that acquires protons from the basic medium environment to maintain pH homeostasis. The design of our device produces very high cell densities at the periphery of the microhabitat array; these very high local cell densities should place GASP mutants at a significant advantage.

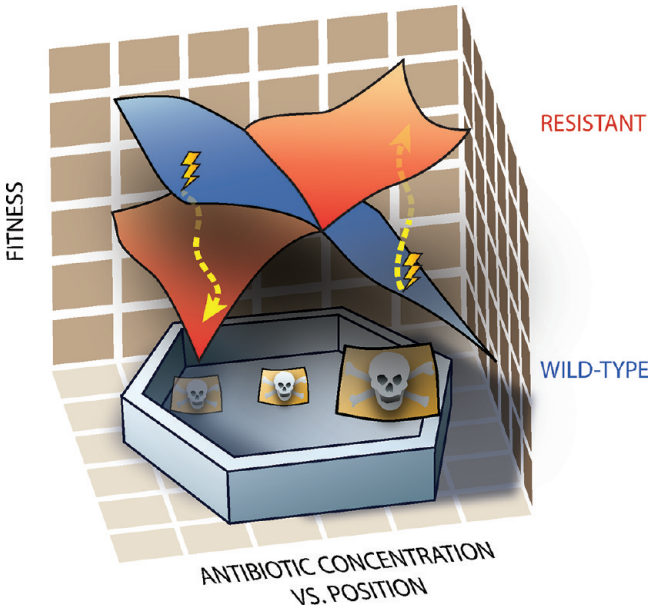

Figure 1. Evolution on nested fitness landscapes. The blue surface and the red surface are the fitness of the wild-type and resistant bacteria respectively vs space in a combination of antibiotic gradient and population gradients. Mutation from the wild-type to the resistant genome is represented by vertical transitions between the two fitness surfaces.

EXPERIMENTAL SECTION

Micronanofabricated structures have been successfully used to study single cells by manipulating their microenvironment,[11−15] but it is still challenging to realize a controllable complex fitness landscape as we seek here. Our previous experiments have been relatively simple one-dimensional (1D) stress experiments.[8] Here, the Galapagos Islands of Darwin have been shrunk down to a 2 cm diameter interconnected network of ∼1200 hexagonal microhabitats, each of a size 200 μm diameter, which can contain up to several thousands of bacteria as shown in the Figure 2. Nutrients circulate around half the perimeter of the device, while nutrients + Ciprofloxacin circulate around the other half of the device, generating gradients of the antibiotic within the array of microhabitats. The nanoslits at the periphery restrict food flow and also can generate emergent population gradients even in the absence of the antibiotic. The 10 μm wide channels connecting the microhabitats allow for the motion of bacteria between different metapopulations, which we will show are a critical aspect of evolution dynamics once stress resistance has evolved in a local metapopulation.

The structure masks were designed in L-Edit and made on a Heidelberg DWL66 laser writer. There are two layers; one contains the microhabitat patches (MHP), while the other contains the nanoslits. The 4" silicon wafer is first spin-coated

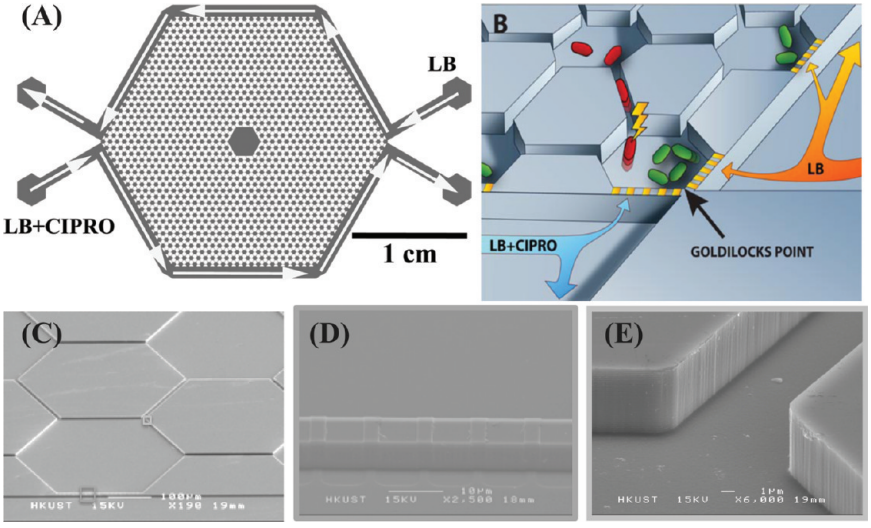

Figure 2. (A) Scheme of microecology consisting of a 2D array of metapopulation. Stable antibiotic gradient is created by flowing nutrient + ciprofloxacin containing streams in one side, while nutrient only stream in the other side. (B) Cartoon of how a Goldilocks point can ignite rapid evolution. (C) ×190 SEM image of hexagonal microhabitat of dimension 200 mm wide (D) ×2500 SEM image of the side of the channel showing 100 nm deep nanoslits (E) ×6000 SEM image of 10 mm deep channel.

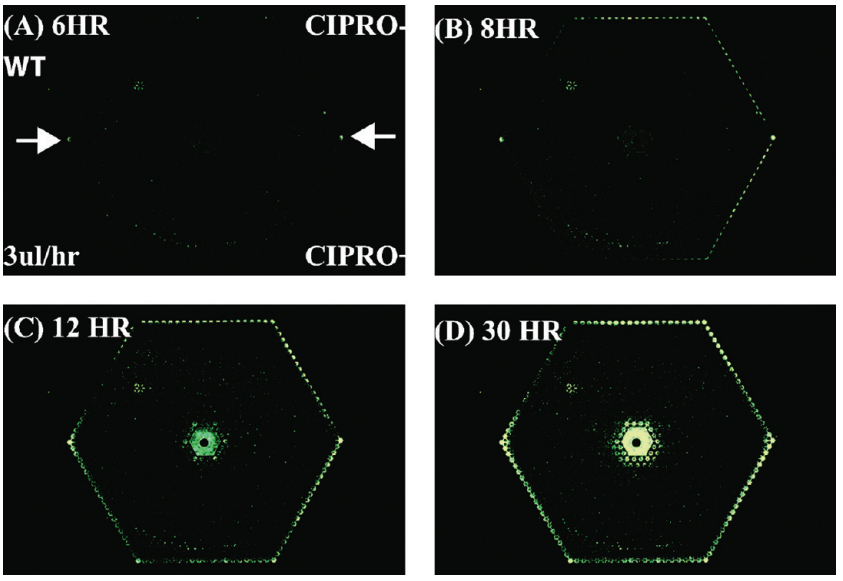

Figure 3. Progression of wild-type *E. coli* vs time after inoculation. (A) Ignition of resistance to Ciprofloxacin at the Goldilocks point (indicated by white arrow) 6 h after inoculation. (B) Spread of resistant bacteria around the periphery of the microecology at 8 h after inoculation. (C, D) Continued growth of resistant bacteria after 10 h.

with photoresist AZ5214, and then the nanoslits mask is exposed by a Karl Suss MA6 mask aligner. After developing in AZ MIF300, 100 nm nanoslits are etched by a reactive ion etching process using a Samco 800 machine. The photoresist is removed by an acetone bath. The process is then repeated with the MHP mask but then etched on a 10 μm thickness.

Our primary choice of antibiotic to show the rapid emergence of the resistance is the important genotoxic antibiotic, Ciprofloxacin.[16] Ciprofloxacin traps the gyrase-DNA complex at the state when the DNA is cut, thereby inhibiting DNA replication and cell division,[17,18] in essence preventing the cell from dividing but not killing the cell. The generation of single-stranded DNA by stalled Ciprofloxacin-bound gyrase is known to trigger, via the self-cleavage of the repressor LexA, removal of LexA from transcription factor sites. Removal of LexA activates the transcription error-prone DNA polymerases.[19] The effective mutagenic rate μ^* due to the SOS response is 10^{-5} mutants/viable cell/day, 10 000 times greater than the base rate μ.[20]

We streaked stock *E. coli* stored at −80 °C onto an agar plate. After overnight growth, one colony was picked and inoculated in a 15 mL tube with 3 mL of lysogeny broth (LB). After growing 3 h, the optical density (OD) reached 0.6/cm. Then 2 μL of the *E. coli* containing medium is gently put at the inlet of center hole, without disturbing the already established antibiotic gradient gradient. Before each experiment, the chip is sealed by a glass coverslip which has spun on it a 20 μm thick elastomer polydimethylsiloxane (PDMS) gasket for sealing. The whole device is held by an acrylic manifold with two inlets and two outlets. The medium is injected with a Chemyx syringe pump. The chip is run with medium and antibiotic alone for 24 h before inoculating bacteria to ensure a stable gradient of Ciprofloxacin + nutrient and nutrient is pre-established. Time lapse fluorescence imaging is realized by Canon 5D camera using a GFP filter set (Chroma). The excitation source is a 470 nm light-emitting diode (LED) from Thorlabs. Images of the entire chip are taken through a Canon 65 mm macrolens by every minutes over 30 h in an enclosure with the temperature control set at 25 °C.

The critical population gradients are emergent and self-generated by the bacterial chemotaxis. If the incoming food flux is much smaller than the local bacterial metabolite consumption rate in a peripheral MHP, the food gradient is self-sharpened by chemotaxis of the bacteria to the edge of the nanoslit source. However, if the incoming food flux is much larger than the bacterial metabolite consumption rate, then we have a spatially uniform food concentration. By simply varying the flow rate, the device can be operated at a desired population gradient over at least 2 orders of magnitude in steepness. Since the antibiotic is not consumed by the *E. coli* and there is continuous flow, the antibiotic gradient, unlike the nutrient gradient, has a constant shape once established.

A noteworthy thought is the importance of the PDMS sealing gasket for the coverslip, which is highly oxygen-permeable. The PDMS layer used for sealing the top coverslip to the chamber actually is important in oxygen transport. That is, a microhabitat with a coverslip anodically hard-bonded to the chamber resulted in no movement of the bacteria to the periphery and no evolution, presumably due to a lack of oxygen.

RESULTS

We have two fundamental results. First, the wild-type evolution dynamics are shown in Figure 3A−D. The emergence and progression of resistance of wild-type *E. coli* at a low-flow rate in each channel of 3 μL/h over 20 h. When bacteria are inoculated into the center of the device, chemotaxis due to the consumption of nutrients at low flow rates quickly drives them to the perimeter of the device to form tight population density gradients against the nanoslits. At the Goldilocks point (white arrow, Figure 3A), there is a combination of high population density gradient and high mutation rates. In this experiment, the concentration of Ciprofloxacin flowing along the bottom side of the device is extremely high, 10 μg/mL, which is approximately 200 times the MIC (minimum inhibitory concentration) of Ciprofloxacin. Yet, as Figure 3A shows, there is an emergence of resistance at the Goldilocks point and subsequent rapid movement of resistant

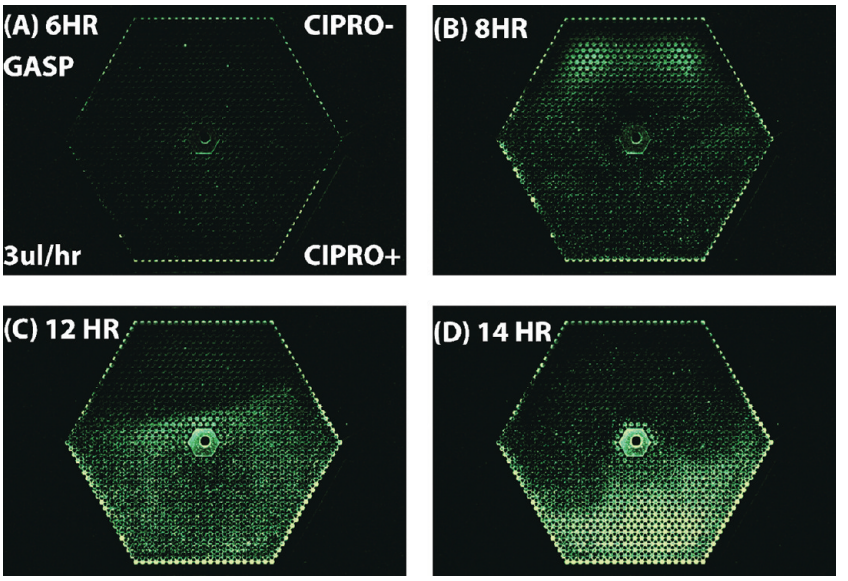

Figure 4. (A–D) Progression of GASP *E. coli* vs time after inoculation. Nutrients flow along the top channel from right to left, while nutrients + 10 μg/mL Ciprofloxacin flow along the bottom channel from left to right.

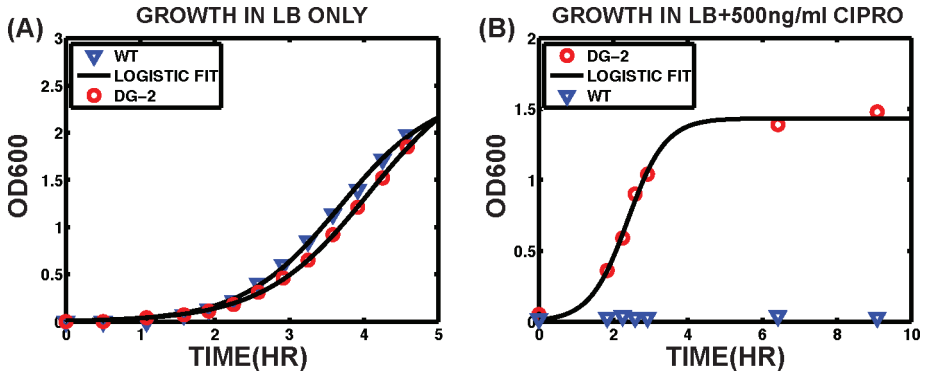

Figure 5. (A) Optical density (OD) vs time of the wild-type (WT) bacteria and the mutants sampled from the high Ciprofloxacin side of the experiments (Figure 3) under standard culture conditions of 37 °C and ×1 LB medium in a test tube. (B) Optical density vs time of the wild-type (WT) bacteria and the mutants under standard culture conditions of 37 °C and ×1 LB medium with 500 ng/mL of Ciprofloxacin added to the test tube.

bacteria around the periphery of the device (Figure 3B) and invasion back to the center in 30 h (Figure 3C,D). The basic reason for this invasion of resistance is the huge fitness advantage for mutant resistant *E. coli* in a microenvironment where the food reservoir is nearby and no other sensitive competitors can live. Since the device is composed of small MHPes, the resistant *E. coli* can rapidly fix in these small populations. The position of the gradients relative to the apex hexagons sensitively determines where the Goldilocks points are.

We expected that the rate and extent to which bacteria were able to colonize our island topography would be a function of the bacterial phenotype; for example, as we stated, the GASP mutants[21] have a considerable fitness advantage to growth under stress and should be able to grow in areas even the wild-type mutants cannot. Figure 4A–D shows the evolution of resistance to Ciprofloxacin at the low flow rate of 3 μL/h. Qualitatively the results are roughly similar: the emergence of resistance at the microhabitat of highest metabolic stress gradient, followed by growth through the device by the resistant bacteria. However, quantitively the evolution of the stress resistance is far more robust and the growth across the device even more extensive than the wild type, showing the fundamental advantage that stress adaption provides for rapid evolution.

That the antibiotic resistance seen in our device is probably due to inherited mutations can be demonstrated by culturing the resistants sampled from the device. This can be shown most dramatically by comparing growth curve of wild-type *E. coli* and *E. coli* sampled from the device in the test tube with the same concentration of Ciprofloxacin. If persisters[22] are the origin of our effect, then the growth curve should be the same for both strains. On the other hand, if the resistants sampled from the device carry inherited mutations, then no growth of wild-type *E. coli* will occur in concentrations where the resistants from the device can now grow. To test this, 3 mL of LB medium containing either resistants from chip or wild-type bacteria were cultured separately in standard 15 mL culture tubes at 37 °C at 500 ng/mL of Ciprofloxacin, ×10 the MIC for wild-type. Growth was measured via the optical density as a function of time. Figure 5B shows that the resistants show growth to high OD under these conditions, while the wild-type's growth is strongly suppressed, as expected.

DISCUSSION

Darwin transformed biology, but he provided no analytical template, and we still have experimentally a great deal to learn about the dynamics of evolution, particularly under response to stress. The actual evolution dynamics occurring in this microecology are no doubt extremely complex and properly viewed as a spatial and temporal convolution with a transport function. However, in the interests of simplicity we can analyze the resistance dynamics in a linear manner here and provide an simple analytical framework. There are several parameters that are relevant to enhancement of the evolution of resistance in mutagenic stress gradients: (1) the N_h: population size in a high stress microhabitat, (2) the stress induced mutation rate μ, (3) the net velocity of bacteria $\vec{v}$ along the gradient, (4) the time before entering the stationary phase τ, (5) the doubling time $t_{1/2}$ of the mutant bacteria in the presence of the antibiotic, and (6) the fitness advantage of the resistant bacteria, s. The time T_e for the adaptive mutants to reach a frequency that assures escape from stochastic loss can be estimated to be:

$$\frac{1}{T_e} = N_h\mu^* + (-\nabla N_h \cdot \vec{v})\tau\mu^* \quad (1)$$

The time to fix T_f for a mutant is expected to scale as:

$$T_f \approx \frac{N_h \times s}{s} \times t_{1/2} \quad (2)$$

The total time T_{total} then expected for the emergence of a fixed population of resistant bacteria is the $T_e + T_f$. We know that N_h is approximately 10^2 and that ∇N_h is $10/\mu$m, τ is about 10^4 s from the test tube culture course measurements, $\vec{v}$ is about 10 μm/s, μ^*(stress induced mutation rate) is about 10^{-5} mutants/viable cells/day. The fitness advantage s is very difficult to estimate and is of course a function of the antibiotic concentration. We can be roughly estimate the ratio of the $MIC_{resistance}/MIC_{wild\text{-}type}$ to be 20. The doubling time $t_{1/2}$ in the presence of the antibiotic at the MIC is not well-known, but we can guess it is approximately 0.5 h. Together, this analysis predicts that T_{total} is approximately 3 h, greatly reduced to the presence of the population gradients and Ciprofloxacin-induced mutations, and is approximately the time observed in our experiments.

CONCLUSION

The rapid evolution of resistance to antibiotics demonstrated in this paper is not just an academic subject of course, although it reveals the importance of understanding the foundations of the dynamics of evolution. The rapid emergence of bacterial resistance to antibiotics has become one of the most important issues in human health. The design principles that we discuss here should provide a powerful template for exploring the rates at which evolution can occur in very complex fitness landscapes, including the rapid evolution of resistance to genotoxic chemotherapy in cancer. While a great deal of work remains to be done to characterize the dynamics of bacterial evolution shown here, the implications of this work we hope are obvious.

For example, the rapid evolution of antibiotic resistance in human bodies is a serious concern. As in our chips, the human ecosystem is very heterogeneous, consisting of solid and liquid components. We would expect that in the body there will exist transient chemical gradients of nutrients and antibiotics. We would expect, based on this work, that in such situations rapid evolution of resistance can occur. Even more alarming is the potential for such rapid evolution to occur during chemotherapy, an area we are now exploring.

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