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Genome Biology

RESEARCH HIGHLIGHT

Whack-an-*E. coli* with the morbidostat

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Abstract

Using a device termed the 'morbidostat', a recent study sheds new light on the determinism of genetic and phenotypic trajectories leading to high antibiotic resistance.

With the discovery of the first antibiotics in the 1940s, conventional wisdom held that the battle against pathogenic bacteria was won. However, shortly after the introduction of these new antibiotics, strains resistant to their action emerged. Today, resistance has been reported even against the latest line of combat embodied by newly developed antibiotics. At this point, it is clear that we do not fully understand the way resistance evolves. Paradoxically, pharmaceutical companies have shut down their basic research aimed at developing new antibiotics because of the low return on investment. In this bleak situation, gaining understanding of the way bacteria evolve antibiotic resistance is crucial.

After decades in which the study of evolutionary trajectories has advanced mainly theoretically, recent years have yielded several studies of *in vitro* evolution in controlled environments, inspired by the pioneering work of Lenski and colleagues [1]. Controlled evolution of parallel bacterial populations in the laboratory has proved to be a tractable system for studying evolutionary trajectories in general [2,3] and antibiotic resistance in particular [4,5]. These studies define new challenges for the theoretical understanding of the way evolution proceeds.

Hammer versus mole

A new study by Kishony and colleagues [6] represents a milestone in the controlled study of evolutionary trajectories of antibiotic resistance in the laboratory. In most studies of *in vitro* evolution, a naïve population is exposed to new conditions and its adaptive trajectory followed over generations. The increase in fitness is rapid at first, as adaptive mutations accumulate, and then slows down when new mutations provide only incremental benefit [3]. In the study by Kishony and colleagues, instead of keeping the chemical composition of the environment constant using a chemostat, the stress inflicted on *Escherichia coli* cultures by the antibiotic was kept constant using an innovative device, the morbidostat. Starting with a low antibiotic concentration, the morbidostat dynamically adjusts the concentration of antibiotic as soon as the culture recovers from the previous concentration, keeping the mean growth inhibition constant. Its action resembles a modified whack-a-mole game (Figure 1). When a mutation appears and confers resistance to the previous concentration, as illustrated by a hat protecting the mole from whacking (Figure 1), a more effective hammer, namely a higher antibiotic concentration, is automatically provided by the computer-controlled morbidostat. This procedure enables scientists to carry out parallel and automated evolutionary experiments under constant evolutionary pressure. One can map the trajectory with which the 'moles' accumulate defense mechanisms in order to survive the constant antibiotic stress.

The trajectories of increasing resistance over time display remarkable phenotypic reproducibility between parallel cultures evolved under the same antibiotic challenge. This reproducibility is exemplified by the evolution of resistance to chloramphenicol (Figure 2). The trajectories for the two other drugs examined, doxycycline and trimethoprim, follow similar dynamics and are also reproducible, although to a lesser degree than for chloramphenicol. This result is fascinating from several perspectives. It enables parameters to be defined that characterize the tempo of evolution for a given antibiotic environment. For example, the trajectories of antibiotic resistance concentration (C) shown in Figure 2 can be fitted to a logistic growth dynamics curve (red) with just two parameters, namely:

$$\frac{dC}{dt} = \mu C\left(1 - \frac{C}{C_{max}}\right) \qquad \text{Equation (1)}$$

where μ is the rate of adaptation and C_{max} is the maximal attainable resistance concentration. C_{max} and μ characterize

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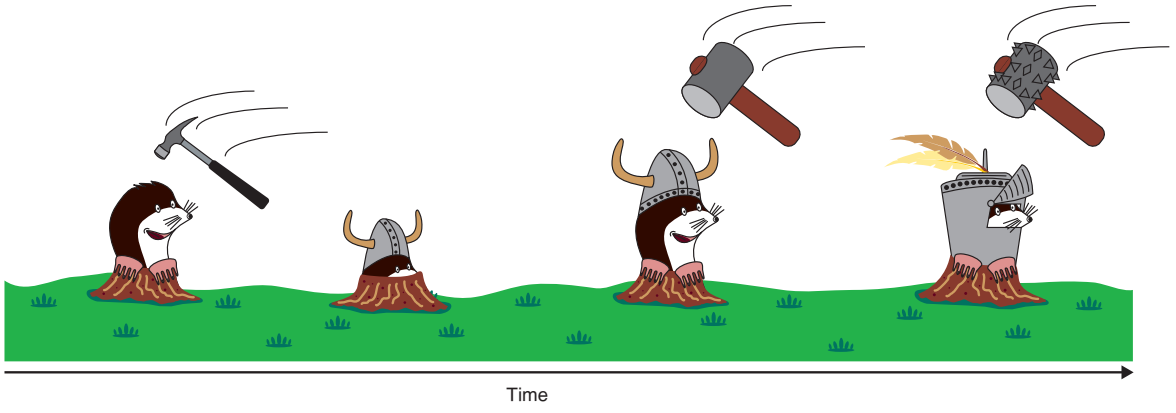

Figure 1. The morbidostat, as a whack-a-mole game, maintaining constant evolutionary pressure despite the evolution of protective mechanisms. An *Escherichia coli* culture is monitored for growth over time. As soon as the culture has grown to exceed a threshold optical density, an antibiotic pulse is added (shown as a hammer whack). If a mutation results in protection from the antibiotic (shown as a protective hat), the antibiotic level is again increased by the computer-controlled morbidostat. As the bacteria develop more protective mechanisms (stronger helmet), the antibiotic level is further increased (heavier/nastier hammer).

the evolutionary trajectory of an antibiotic. It is highly probable that apart from its dependence on the antibiotic chosen, μ also depends on parameters governing evolution, such as effective population size and mutation rate, whereas C_{max} is characteristic mainly of the antibiotic itself. For example, trimethoprim and chloramphenicol have C_{max} values that are three orders of magnitude higher than the initial half-maximal inhibitory concentration (IC_{50}), whereas for doxycycline this parameter is only one order of magnitude higher. Once these parameters are known, they enable quantitative comparisons between different antibiotic regimes, for example different drug combinations. Indeed, the Kishony laboratory has demonstrated, in a series of clever studies, that resistance to drug combinations can work in a counterintuitive manner [7]. Minimizing the rate of adaptation to a drug combination is an important clinical consideration, and the morbidostat provides a unique way to assess and compare adaptation rates. The universality of such parameters when characterizing a drug treatment remains to be determined: do they depend on local morbidostat settings or, instead, do they reveal some more inherent evolutionary factors?

Genotypic trajectories

The reproducibility of the resistance trajectories between parallel cultures seems to suggest that the underlying genetic trajectories are also similar. Making use of recent advances in whole genome sequencing (WGS), Kishony and colleagues characterized the end points of the parallel trajectories for all five replicates for each of three antibiotics. Whereas resistance mutations to trimethoprim were reproducibly located mainly in the target gene *DHFR* (encoding dihydrofolate reductase), diverse genetic alterations were found to underlie the strikingly similar trajectories for chloramphenicol (Figure 2). This notwithstanding, the majority of mutations observed under both chloramphenicol and doxycycline treatment converge mainly on one goal, namely decreasing the internal drug concentration by activating efflux, or decreasing influx, both under the control of the multiple drug resistance pathway [8]. The authors analyzed further the reproducibility in the evolution of resistance to trimethoprim by Sanger sequencing the *DHFR* gene over time. This analysis revealed a typical accumulation order of the different single nucleotide mutations, indicating that the underlying genetic trajectory is reproducible.

Globally, the genes identified by WGS are not surprising: two-thirds of the genes identified are either direct targets of the antibiotics or genes involved in the multiple drug resistance pathway. The study identifies *yedX*, *lpxM*, *manY* and *isrC* as potential new players in the drug resistance game. However, the significance of the sequence data goes far beyond the mere identification of a handful of new genes. When considering the evolution of bacteria, one must bear in mind that, in the absence of recombination or sexual mating, selection forces are applied on the bacterial genome as a whole. A genome-wide perspective is therefore essential to the analysis of adaptive processes. WGS theoretically paints a complete picture of all the genomic differences between an evolved strain and the wild-type reference. Yet, it remains unclear if the entire evolutionary change is indeed unveiled by WGS, an issue that can be addressed by recreating the observed fitness of the evolved strains using allelic replacement [9]. For example, it becomes clear that SNPs are not the sole contributors to genomic variation. Over the evolutionary process, the bacterial

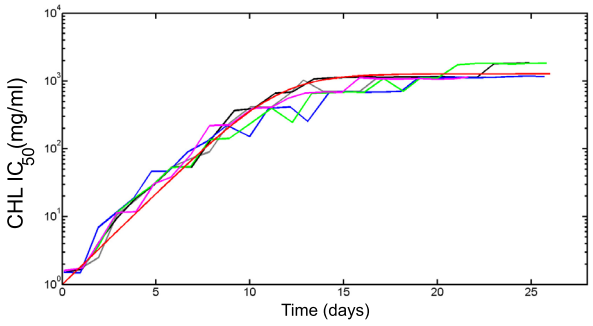

Figure 2. Reproducibility of parallel evolution of antibiotic resistance. Resistance levels over time for five parallel populations evolving in morbidostats under a dynamic chloramphenicol (CHL) challenge (adapted with permission from Toprak *et al*. [6]). The red line is a logistic curve fit according to Equation 1.

genome accumulates various types of chromosomal alterations, and their characterization is of uppermost importance for genome reconstruction. For example, gene duplication-amplification (GDA) events are among the most common types of mutations, occurring with a frequency of between 10^{-2} and 10^{-4} per cell [4]. Previous studies have shown that bacteria can obtain resistance to different drugs, including chloramphenicol and trimethoprim, by duplicating various loci in their genome [10]. A notable feature of GDAs is that they can be lost just as quickly as they appear, making this alteration unstable, especially when selection is removed. In the context of drug resistance, GDAs could grant bacteria transient resistance, thus allowing them to survive until a slower *de novo* adaptation appears in their genome, at which time the GDA could disappear. In the study of Kishony and colleagues, GDA was observed in only 4 out of the 19 strains that were sequenced; this is a low occurrence rate given earlier observations [4]. This discrepancy could be interpreted as indicating that GDAs do not play a pivotal role in the scenario of dynamically sustained drug selection, or alternatively it could be due to their aforementioned fickle nature. Improvements in the current technologies and analyses will need to be applied to overcome the difficulties facing genome reconstruction. Furthermore, WGS of frozen intermediate evolving cultures could assist in capturing transient events such as GDAs. In future studies, the decreasing costs of paired-end sequencing and *de novo* assembly, along with the ever increasing quality and coverage of reads, should allow us to identify various types of chromosomal alterations (including GDAs, insertion elements, inversions and deletions), and also to fully characterize and localize these alterations within the genome.

In summary, the work of Kishony and colleagues represents an important advance in the way *in vitro* evolutionary experiments are conducted. Together with recent analyses of antibiotic resistance evolution under controlled conditions [5], their study paves the way forward for the development of new approaches that follow the evolution of antibiotic resistance in a reproducible way. Finally, experimental strategies similar to the morbidostat method could shed light on the evolution of drug resistance in cancer cells; this is another alarming example of rapid and aggressive asexual evolution under constant pressure, and which also has broad clinical consequences.

Abbreviations

GDA, gene duplication-amplification; SNP, single nucleotide polymorphism; WGS, whole genome sequencing.

Competing interests

The authors declare that they have no competing interests.

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