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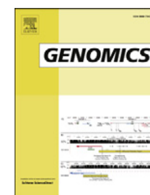

The enduring utility of continuous culturing in experimental evolution

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ABSTRACT

Studying evolution in the laboratory provides a means of understanding the processes, dynamics and outcomes of adaptive evolution in precisely controlled and readily replicated conditions. The advantages of experimental evolution are maximized when the selection is well defined, which enables linking genotype, phenotype and fitness. One means of maintaining a defined selection is continuous culturing: chemostats enable the study of adaptive evolution in constant nutrient-limited environments, whereas cells in turbidostats evolve in constant nutrient abundance. Although the experimental effort required for continuous culturing is considerable relative to the experimental simplicity of serial batch culture, the opposite is true of the environments they produce: continuous culturing results in simplified and invariant conditions whereas serially diluted batch cultures are complex and dynamic. The comparative simplicity of the selective environment that is unique to continuous culturing provides an ideal experimental system for addressing key questions in adaptive evolution.

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1. Introduction

Experimental evolution with microbes commenced at least 130 years ago with the work of Darwin's contemporary, Reverend W. H. Dallinger [1]. However, for many years progress in experimental evolution was limited by the inability to comprehensively characterize the genetic variation associated with adaptive evolution. The advent of genomic technologies solved this problem, first through the use of DNA microarrays to identify nucleotide [2,3] and structural [4] variation, and subsequently with the application of quantitative high throughput DNA sequencing [5–9]. Whole genome sequencing of both individual lineages and entire populations is no longer a roadblock to progress, and has rapidly become a routine experimental method that has transformed the field of experimental evolution. These technological advances mean that many long-standing questions in evolutionary biology can now be addressed with unprecedented detail, precision and rigor.

The dawn of a new era in experimental evolution warrants revisiting the major goals of the research program of experimental evolution. These goals have been discussed in recent publications [10,11], including those accompanying this article, and can be summarized as follows: 1) understanding the molecular basis of adaptation at the functional and mechanistic level, 2) understanding the consequences of adaptive mutations on organismal phenotypes and physiology, 3) defining the predictability and repeatability of adaptive evolution, 4) mapping the distribution of fitness effects of mutations, 5) determining how parameters such as population size and strength of selection affect adaptation, and 6) identifying the parameters that affect the dynamics of adaptive evolution.

In general (but not exclusively [12,13]), experimental microbial evolution entails selection of *de novo* mutations that arise in an initially genetically clonal population. Thus, experimental evolution in microbes differs from experimental evolution in animals such as worms [14], flies [15] and mice [16], which typically entails selection on standing (pre-existing) genetic variation by founding populations with genetically heterogeneous individuals. When undertaking experimental evolution with microbes, the ease of maintaining large populations (10^8–10^{10} individuals) with short generation times (20–360 min) that typically have small genome sizes (10^6–10^7 bases) with typical mutation rates of 10^{-7}–10^{-9} substitutions/bp/generation means that mutation supply is extremely high. In many experimental evolution scenarios it is reasonable to assume that on average every possible one base substitution in a microbial genome is introduced into the population each generation. Thus, selection has ample diversity on which to act.

Technically, experimental evolution with microbes entails selection over prolonged periods of culturing in laboratory conditions. This can be achieved by simply passaging cells in culture flasks (i.e. batch cultures) using the method of serial transfer. For the practiced experimentalist there are few microbiology techniques that are simpler than transferring a sample from one population to inoculate a new culture containing fresh medium and thus initiate a new round of population growth. Moreover, the method of serial dilution of batch cultures is readily

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amenable to parallelization using microtiter plates and robotic liquid handling, which enable the simultaneous analysis of hundreds of populations [9,17].

Alternatively, long-term selection can be performed using methods of continuous culturing including chemostats and turbidostats. In contrast to serial transfer of batch cultures, long term selection using continuous culture can be logistically challenging and less amenable to large-scale multiplexing, leading to the reasonable question: "why bother?" The goal of our article is to argue that the answer to this question lies in the great utility of maintaining a continuous and invariant selection during experimental evolution. Continuous culturing, using chemostats or turbidostats, provides the only means of ensuring a sustained and invariant selective pressure, a feature that greatly simplifies the goal of connecting adaptive genotypes with their phenotypic consequences and explaining why they result in increased fitness. As a result, continuous culturing is ideally suited to addressing some of the central goals of experimental evolution.

2. The principle of the chemostat

The principle of the chemostat differs in several respects from batch culture [18]. In a chemostat, fresh medium is continuously added to the growing culture at a defined rate and at the same rate culture is removed. Eventually, the culture reaches a steady-state in which the cells grow continuously at a constant rate and the growth rate of the population is equal to the rate at which it is diluted [19,20]. Through the process of continuous dilution a growing population of cells can be maintained in a chemostat indefinitely. An essential requirement of the chemostat is the use of a defined medium in which a single nutrient is present at a growth limiting concentration [21]. A nutrient is said to be limiting in the chemostat when its concentration dictates the steady-state cell density, such that increasing the concentration of the limiting nutrient results in a proportional increase in the steady-state cell density. In the steady-state condition the concentration of the growth-limiting nutrient is typically in the low micromolar range. Thus, cells in a chemostat grow continuously in a chemically defined environment where all nutrients but one are present in excess. This environment is most similar to a batch culture just prior to nutrient exhaustion and has been described as placing the cells in an environment in which they are "poor, not starving" [22] or "hungry" [23]. The low concentration of the growth-limiting nutrient defines the selection imposed on cells. A variety of growth-limiting nutrients can be used, so long as they are essential for growth of the organism. Typically, these are sources of carbon, nitrogen, phosphorous or sulfur, though non-essential nutrients can also be made essential by the use of appropriate auxotrophic mutants that are defective in a biosynthetic pathway. Increases in fitness in the chemostat environment are typically achieved by improved capabilities in the acquisition or utilization of the growth-limiting nutrient.

3. The principle of the turbidostat

A turbidostat is analogous to a chemostat in that the culture is continuously diluted by the addition of fresh medium. However, in contrast to a chemostat, the goal of a turbidostat is to avoid cells ever experiencing nutrient limitation. This is achieved by continuous addition of fresh medium to the growing culture to maintain a specific cell density. As with a chemostat, the culture is continuously diluted by the addition of medium and the removal of an equivalent volume culture. However, in the case of a turbidostat, all nutrients are present in excess and the dilution rate is set near the maximal growth rate of the cells. In practice this is achieved by constant monitoring of cell density and automated addition of media when the density exceeds the specified value. The resulting steady-state environment is most similar to a batch culture during the mid-log exponential phase of growth, when growth rate is maximal, and nutrients are in abundant supply.

Unlike a chemostat, the growth rate of cells in a turbidostat is determined by intrinsic properties of the cell. As the turbidostat environment is never nutrient poor, the ability of cells to grow is not constrained by nutrient abundance. Instead, the limits to growth are inherent properties of the cell that determine how rapidly it can replicate. Factors that likely limit the rate a cell can reproduce itself when resources are abundant include the rate of nutrient uptake and the rate of macromolecular and organelle biogenesis, as well as complex molecular processes such as DNA replication, transcription and translation. In principle, increases in fitness in the turbidostat might result from enhancements in any of these processes. Variants on turbidostats include devices in which the ability to grow maximally is constrained by an environmental agent, for example by adding growth inhibitors such as high ethanol or antibiotics [24,25].

4. Distinction from serial transfer in batch cultures

Despite the very different selective pressures that operate in the chemostat compared with a turbidostat, both methods share the principle of continuous culturing and therefore a continuous selection. A comparable constancy of selective pressure is not possible using serial dilution of batch cultures, even when great care is taken to transfer from exponentially growing cultures prior to the onset of stationary phase [26,27]. Regardless of whether an undefined medium is used, in which the environmental factor that determines the population size at the end of each growth phase is unknown, or a defined medium in which the nutrient that is first exhausted and therefore determines the final population size is pre-determined, dramatically fluctuating levels of nutrient abundance are characteristic of experimental evolution using serial transfer (Fig. 1). In fact, a batch culture of cells experiences both a turbidostat-like and chemostat-like environment during each growth cycle in addition to experiencing near or complete starvation, depending on the period length of the transfer cycle.

It is certainly true that the repeated cycles of feast and famine in a serial transfer experiment impose a strong selection on cells. However, at this point we do not understand which phase of the growth cycle is the predominant selective force in a serial passage regime, and the relative importance of factors, such as nutritional abundance, intracellular processes and excreted products, is likely to change over the course of a single passage during a serial dilution evolution experiment. As a result, increased fitness in batch culture may result from decreased duration in lag phase (the time taken to reinitiate growth upon encountering fresh medium), increased growth rate during the growth phase or a decreased probability to enter a quiescent, non-reproductive, state upon nutrient depletion [28,29]. It is quite plausible that alleles that improve fitness in each of these growth phases are antagonistically pleiotropic with respect to each other. Thus, allele frequencies may fluctuate throughout each serial passage or different lineages may specialize in optimizing one or more of each of the phases of batch culture growth. Continuous culturing provides a means of avoiding this complexity. The constancy of selection in a chemostat or turbidostat enables the selection to be precisely defined and indefinitely maintained providing considerable advantages for addressing the following key questions using experimental evolution.

5. What is the molecular basis of adaptation?

Determining the molecular basis of adaptation is critical for advancing understanding in evolutionary biology [30]. By understanding the mechanistic basis of adaptation we can begin to explain why particular outcomes of adaptive evolution are favored over other possibilities. For example, adaptation in some selective environments may entail alteration of a single biochemical pathway or protein complex whereas

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