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Original Article

Adaptation of *Escherichia coli* to long-term batch culture in various rich media

Lacey L. Westphal [a], Jasmine Lau [b], Zuly Negro [b], Ivan J. Moreno [b], Wazim Ismail Mohammed [c], Heewook Lee [d], Haixu Tang [c], Steven E. Finkel [a], Karin E. Kram [b, *]

[a] Molecular and Computational Biology Section, Department of Biological Sciences, University of Southern California, RRI 303, 1050 Child's Way, Los Angeles, CA, 90089-2910, USA
[b] Department of Biology, California State University, Dominguez Hills, NSM A-137, 1000 E. Victoria Street, Carson, CA, 90747, USA
[c] School of Informatics and Computing, Indiana University, 150 S. Woodlawn Avenue, Bloomington, IN, 47405, USA
[d] Computational Biology Department, School of Computer Science, Carnegie Mellon University, GHC 7719, 5000 Forbes Ave., Pittsburgh, PA, 15213, USA

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ABSTRACT

Experimental evolution studies have characterized the genetic strategies microbes utilize to adapt to their environments, mainly focusing on how microbes adapt to constant and/or defined environments. Using a system that incubates *Escherichia coli* in different complex media in long-term batch culture, we have focused on how heterogeneity and environment affects adaptive landscapes. In this system, there is no passaging of cells, and therefore genetic diversity is lost only through negative selection, without the experimentally-imposed bottlenecking common in other platforms. In contrast with other experimental evolution systems, because of cycling of nutrients and waste products, this is a heterogeneous environment, where selective pressures change over time, similar to natural environments. We determined that incubation in each environment leads to different adaptations by observing the growth advantage in stationary phase (GASP) phenotype. Re-sequencing whole genomes of populations identified both mutant alleles in a conserved set of genes and differences in evolutionary trajectories between environments. Reconstructing identified mutations in the parental strain background confirmed the adaptive advantage of some alleles, but also identified a surprising number of neutral or even deleterious mutations. This result indicates that complex epistatic interactions may be under positive selection within these heterogeneous environments.

1. Introduction

Experimental evolution systems using *Escherichia coli* have begun to address many fundamental questions regarding mechanisms of evolution, including the methods by which organisms adapt to stressful conditions, the mutational pathways that allow adaptations to specific responses, whether evolution always follows the same path for a given environment, and how a changing environment may affect the evolutionary trajectory of bacteria. Because the evolutionary process is complex, most previous experiments have been conducted in well-defined, relatively simple environments.

Among the most well-known experimental evolution experiments using bacteria is the serial passage regime pioneered by Richard Lenski and colleagues [1–3]. This system has several key characteristics: 1) populations of cells are incubated in a defined, minimal medium containing glucose as the primary carbon source, 2) every 24 h, after ~7 generations of growth, 1% of the population is transferred into a fresh culture, and 3) populations are frozen every 500 generations to use as "time capsules" to track evolutionary trajectories. Fitness coefficients can be calculated by competing evolved strains with the parent strain and determining relative growth yields. The selective pressure here is fairly well understood: mutants that can grow faster and/or to a higher density generally

* Corresponding author.
E-mail addresses: llwestphal@gmail.com (L.L. Westphal), jlau7@toromail.csudh.edu (J. Lau), znegro1@toromail.csudh.edu (Z. Negro), imoreno24@toromail.csudh.edu (I.J. Moreno), wazimoha@umail.iu.edu (W. Ismail Mohammed), heewooklee@gmail.com (H. Lee), hatang@indiana.edu (H. Tang), sfinkel@usc.edu (S.E. Finkel), kkram@csudh.edu (K.E. Kram).

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have increased their ability to metabolize glucose, or citrate in later experiments [1,2,4,5]. Variations of this system include using different bacterial species [6,7], increasing the amount of time cultures are grown in a given environment [8], alternating nutrient sources [9], and exposing populations to other stresses including heat, antibiotics, or ionizing radiation [10–21].

In a different type of evolution experiment, in order to keep growth conditions constant, other groups have cultured populations using chemostats or continuous culture systems [22–24]. The chemostat ensures a constant environmental condition is maintained, but also introduces loss of population diversity as cells and spent medium are continuously flushed out of the system.

A third type of experimental evolution system, referred to here as a "long-term batch culture" system, has been used to study heterogeneous and changing environments by evolving populations in complex media through long-term stationary phase [25–31]. In this system, cells are inoculated at initiation of the experiment, and then no further manipulations of the cells or environment are performed until the conclusion of the experiment. In batch culture systems, any bottlenecking that occurs is part of the natural population dynamics of the culture, not through experimental manipulation, and therefore all changes observed are due to the growth and death of cells in the population; the environment is constantly changing and the population retains genetic diversity that has accumulated over the course of the experiment. This cell growth and death during long-term stationary phase contributes to cell survival by providing novel nutrients to which the cells can adapt [32]. Because the environment that these populations encounter changes over time, fitness landscapes are less predictable [33]. For example, populations that have been incubated for 1 day in batch culture may experience a strong selective pressure for mutants that have fortified stress resistance mechanisms to changes in pH or oxygen availability, such as mutations that cause attenuation of RpoS, a stationary phase sigma factor [31,34]. However, populations in longer-term cultures may experience completely different selective pressures where a mutation that allows for the metabolism of non-preferred carbon sources may confer a greater advantage than those that help with more short-term stresses [8,27].

While the exact selective pressure(s) in these types of environments are not fully understood, we know that evolution is occurring because of the appearance of mutants expressing the Growth Advantage in Stationary Phase (GASP) phenotype, characterized by the ability of cells from aged populations to out-compete un-aged parental strain populations [26,29,31,34]. While *E. coli* can survive in batch culture for days, weeks, or even years without the addition of nutrients [26,31,34,35], experiencing the five phases of growth (lag phase, log phase, stationary phase, death phase, and "long-term stationary phase" [LTSP]), it isn't until LTSP that GASP mutations are first observed [31,34–36]. During batch culture incubation, survival of cells better suited to their particular environment leads to selection of beneficial alleles and therefore evolution of these populations [26,27,31,34]. Further, because populations that continue to be aged (e.g., for 20 or 30 days) continue to produce mutants with increased relative fitness, we have hypothesized we are not observing single sweep events, but instead multiple subpopulations harboring beneficial mutations are present in the culture at any given time [31]. Interestingly, when populations are grown in different rich media or under different culture environments, they experience different levels of glycation stress, pH stress, and mutation frequencies [37,38], which led us to hypothesize that the adaptive pathways in different rich media will vary.

Using each of the experimental evolution systems, a number of phenomena other than the GASP phenotype have also been observed. Many positively selected mutations have been detected in genes encoding regulator proteins, including *rpoS* [3,20,31,34,39–42], *rho,* and *rpoBC* [13] where a single mutational event can broadly alter the physiology of a cell. Several groups have also found diminishing returns epistasis where initial mutations cause relatively large increases in fitness, but over time further adaptive mutations result in smaller fitness gains [3,20,39–41]. It is hypothesized that initial mutations in regulators can cause large increases in fitness and further mutation serves to mitigate or fine-tune the adaptive response to the first mutation [13,43]. To explore whether these observed phenomena occur across multiple environments, we conducted experimental evolution studies within a long-term batch culture environment by evolving *E. coli* in various rich media for 10 days, enough time to accumulate GASP mutations in Luria–Bertani Broth (LB).

To determine how different rich medium environments influence the adaptation of *E. coli*, we aged populations in four different media (Luria–Bertani, LB; 2x Yeast Extract-Tryptone, YT; Terrific Broth, TB; and Super Broth, SB), and assessed whether they accumulated adaptive mutations to their environment within 10 days, the time required for cells to express the GASP phenotype in LB [34]. This experimental approach allowed us to study several aspects of evolution: 1) the speed of evolution: Does the environment affect how quickly cells adapt to LTSP?; 2) the "generality of adaptation": Are mutations that have been selected in one environment also beneficial in another environment?; and 3) evolutionary parallelism: Do similar adaptive mutations arise within and/or between populations in different environments? The answers to these questions address a fundamental aspect of evolution, that is, how do divergent environments affect evolutionary trajectories? We find that the environment affects both the timing of evolution and the adaptive pathways. While there is some overlap between adaptive phenotypes and adaptive genotypes among the four environments, we also observe differences among the mutations identified in each environment. These results indicate that even subtle differences in the environment can alter the adaptive evolutionary trajectories of populations.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and viable cell counts

An *E. coli* K-12 lineage strain, MG1655-derivative PFM2 [44], and its streptomycin resistant (str^R), kanamycin resistant (kan^R) and chloramphenicol resistant (cam^R) derivatives described below, were used in this study. For competitions (described in detail below), parental strains were transduced with a chloramphenicol cassette inserted into *lacZ* (*lacZ*:cam^R), and reconstructed mutant strains were transduced with a kanamycin resistance cassette inserted into *lacZ* (*lacZ*:kan^R) from strains previously constructed with these alleles using the Datsenko-Wanner method [45]. Frozen 20% glycerol stocks of parental cultures were saved from overnight cultures initiated from a single clone. Overnight cultures were initiated from frozen stocks and inoculated into 5 ml of Luria–Bertani (Lennox) medium (LB) (Difco) in 18- by 150-mm borosilicate test tubes and were incubated with aeration in a TC-7 rolling drum (New Brunswick Scientific, Edison, NJ) at 37 °C [38]. All aging cultures were inoculated from the same overnight outgrowth. For population sampling, 100 μl of cells were removed from aging cultures and saved in 20% glycerol stocks on days 1, 2, 4, 6, 8, and 10 from triplicate cultures incubated in each medium (Luria–Bertani broth (LB), 2x Yeast Extract-Tryptone (YT), Terrific Broth (TB), Super Broth (SB)). Cell growth and survival was monitored as described previously [38]. Briefly, cells were inoculated from overnight cultures at a 1:1000 (vol:vol) dilution and serially