

Revisiting the Design of the Long-Term Evolution Experiment with *Escherichia coli*

Richard E. Lenski^{1,2}

ORCID 0000-0002-1064-8375

✉ lenski@msu.edu

¹ Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824, USA

² Ecology, Evolution, and Behavior Program, Michigan State University, East Lansing, MI 48824, USA

Abstract

The long-term evolution experiment (LTEE) with *Escherichia coli* began in 1988 and it continues to this day, with its 12 populations having recently reached 75,000 generations of evolution in a simple, well-controlled environment. The LTEE was designed to explore open-ended questions about the dynamics and repeatability of phenotypic and genetic evolution. Here I discuss various aspects of the LTEE's experimental design that have enabled its stability and success including the choices of the culture regime, growth medium, ancestral strain, statistical replication, and the like. I also discuss some of the challenges associated with a long-running project, such as handling procedural errors (e.g., cross-contamination) and managing the expanding collection of frozen samples. The simplicity of the experimental design and procedures have supported the long-term stability of the LTEE. That stability—along with the inherent creativity of the evolutionary process and the emergence of new genomic technologies—provides a platform that has allowed talented students and collaborators to pose questions, collect data, and make discoveries that go far beyond anything I could have imagined at the start of the LTEE.

Keywords. Evolutionary dynamics · Experimental design · Genome evolution · Microbial evolution · Relative fitness

Introduction

The Long-Term Evolution Experiment, or LTEE for short, has been productive in three respects. First, the LTEE has produced many new findings about the dynamics and outcomes of evolution. I will not review those discoveries here, because they have been reviewed elsewhere (Lenski 2004; Lenski 2017a). However, I will mention some of them that bear on specific issues of experimental design. Second, the LTEE has provided a fertile training ground for many talented undergraduate students, graduate students, and postdoctoral researchers who have worked in my laboratory and the laboratories of collaborators around the world. Third, the LTEE has served as a model for other researchers interested in designing and performing experiments to study evolutionary processes,

in general, and microbial evolution, in particular. With that last point in mind, the editors of this collection of papers asked me to address the following question: If I were setting up a similar experiment today, would I do things differently?

Let me begin by saying that scientists have their own preferences and predilections when it comes to research. These preferences are evident not only in the scientific issues we study, but also in the ways we go about addressing those issues. In my case, I am usually drawn, at least at the start of a project, to abstract questions, rather than to particular organisms or the mechanistic details that underlie phenotypes (Fox and Lenski 2015). I also prefer to undertake projects that will likely generate useable data, as opposed to ones that are entirely new and might fail completely. That is not to say that I set out to confirm or refute some hypothesis; instead, I am curious about what the data might show, and I want to be confident that relevant data can be obtained. In that same vein, I try to keep the design of a study as simple as feasible, both methodologically and conceptually. That simplicity leaves fewer opportunities for procedural errors, and it also often makes the resulting data easier to interpret and explain. Last but not least, it is extremely helpful to work with talented collaborators and students, and to give them the latitude to explore new technologies and questions, while also ensuring that the project as originally planned continues to progress. The reason I discuss these predilections is to state clearly, at the outset, that there is not a single best way to do science, in general, or experimental evolution, in particular. Rather, there are many ways of doing good science, and the ways that are best for each scientist will depend on individual preferences. In other words, “To thine own self be true.”

Questions

Before embarking on an evolution experiment (or any experiment, for that matter), one presumably has some questions that one would like to answer. Some questions are specific and focused, while others are general and even open-ended. In the case of the LTEE, I had three broad questions—or really, three sets of questions—in mind.

How repeatable is evolution? The repeatability of evolution has long been of interest to many biologists, given the fundamental tension between deterministic forces, primarily natural selection, and stochastic processes, including mutation and genetic drift. The LTEE allows us to examine the repeatability of evolution by having 12 replicate populations, all founded from the same ancestral strain and all propagated in the same environment. One possibility is that evolution—especially adaptive evolution—is repeatable owing to the power of natural selection to find similar solutions to the same environmental challenges and opportunities. Alternatively, evolution might be highly unpredictable and idiosyncratic, with each solution depending on the random nature of mutations and the contingent history of the evolving system to that point. Of course, the extent of repeatability is unlikely to be the same for all traits, and so one can ask: Which phenotypic and genomic changes are repeatable, which are haphazard, and what determines the extent of parallelism or divergence for a given trait?

What are the dynamics of evolution? A second set of questions for the LTEE concerns the tempo and mode of evolutionary change. Is evolution invariably gradual, or are there episodes of more rapid change even in a constant environment? How do these dynamics depend on which phenotypic or genetic traits are measured? In the case of fitness, does improvement continue indefinitely, or is some limit eventually reached? One possibility is that phenotypic evolution is invariably slow and gradual because it involves many changes with each one having a small effect.

Alternatively, phenotypic evolution might sometimes exhibit punctuated dynamics resulting from the rapid substitution of mutations of large effect. Once again, these are questions of long-standing interest, and the answers are likely to depend on the specific phenotypic and genetic metrics that one examines. The LTEE allows us to address these issues by providing a detailed time-series of evolutionary changes.

How are phenotypic and genomic evolution coupled? The third set of questions explores the integration of phenotypic and genetic changes. Are the dynamics of phenotypic and genomic evolution concordant in time? If phenotypic traits evolve in parallel across replicate populations, does this imply parallelism at the level of mutations, genes, or pathways? Will the bacteria become ecological specialists as they adapt to their constant environment? If so, is specialization driven by pleiotropic tradeoffs or the accumulation of neutral mutations? The capacity to freeze and revive bacteria from throughout the LTEE, and the ability to find and manipulate mutations, facilitates the rigorous and precise integration of phenotypic and genetic data. However, when the LTEE began in 1988, no genomes had been sequenced for any organism, and it was costly even to sequence individual genes. New technologies have greatly expanded our capacity to pursue this integration.

Background

Many projects are started only after some preliminary work has been performed. In the case of the LTEE, I had previously run an experiment that was almost identical to the LTEE, except in two respects (Lenski 1988). First, the earlier experiment had run for only 400 generations. Second, it compared fitness trajectories in two sets of *Escherichia coli* populations; one set was resistant to bacteriophage T4, while the other set was sensitive. The sensitive populations served as a control for quantifying the extent of compensatory evolution to reduce the pleiotropic costs of resistance. The sensitive progenitor later became the ancestor of the LTEE. Otherwise, the basic procedures and environment that we use in the LTEE are identical to the earlier experiment.

That earlier experiment provided some important lessons, and it also raised new questions in my mind. First, it showed that it was feasible to keep a serial-transfer experiment going indefinitely without unreasonable effort (aside from some work on weekends and holidays) and without major problems. Second, it confirmed that we would see significant fitness gains within that timeframe. However, I also suspected that only one or two mutations had swept to fixation over that period. Because I was interested in fitness landscapes and how their shapes would affect the repeatability of evolution, I realized that I should perform a longer experiment that would allow more genetic evolution.

With that in mind, and after discussing these issues with Michael Rose (my then colleague at UC-Irvine), I decided to repeat that experiment with two differences. First, the new experiment would run longer to allow more successive mutations to occur, so that the evolving populations could explore the fitness landscape more widely. Second, all of the populations would start from the same ancestral strain, thereby providing a larger sample size for making inferences about the emergence of variation between populations from initially identical genotypes. The original goal was to run this experiment for 2000 generations, which I anticipated would allow time for several selective sweeps in each population, and which I thought would deserve to be called a long-term evolution experiment. Indeed, an analysis of fitness trajectories in the first LTEE paper suggested there were, on average, ~3.7 selective sweeps per population during the first 2000 generations

(Lenski et al. 1991). Whole-genome sequences obtained much later indicated that the bacteria had accumulated, on average, ~ 7.5 mutations in those 2000 generations, with compelling evidence that many of them were beneficial “driver” mutations (Tenaillon et al. 2016).

Experimental Design

I will now discuss some of the important considerations and choices with respect to the design of the LTEE and the specific procedures used. (As I explained above, many of these choices were made in the experiment that proceeded the LTEE.) I will explain the choices I made, some of the alternatives I considered, and why the choices mattered for the LTEE. I think these issues are worth careful consideration by anyone who is considering a new microbial evolution experiment.

Culture regime. The LTEE uses a serial-transfer regime. Each day, we transfer 0.1 mL of the previous culture into 9.9 mL of fresh medium. Both volumes are easily measured using standard microbiological tools, making the transfer procedure simple and repeatable. After each transfer, the bacteria experience a sudden abundance of resources, and the population grows until the limiting resource is depleted, at which point the cells enter stationary phase. The 100-fold regrowth of the bacterial population corresponds to $\log_2 100 \approx 6.6$ cell generations per day. There is little or no cell death after the resources have been depleted in the LTEE. However, death might become important for other species, in different media, or if the period between transfers was much longer.

An alternative regime would maintain the populations in a chemostat, where they would grow at a constant rate maintained by continuously adding fresh medium and removing an equal volume (including cells in the volume that is removed). A chemostat has several potential advantages, at least in principle. First, it is not necessary for someone to perform the daily transfers. Instead, one needs only occasionally to refill the reservoir that provides fresh medium. Second, the bacteria are growing at a constant rate that equals the rate of turnover of the medium. This constant growth rate might make it easier to dissect the demographic and physiological processes responsible for adaptation to the experimental environment. Third, the constant growth in a chemostat makes it easier to model evolution mathematically, including the differential growth of competing lineages in a population. Fourth, one would avoid the bottlenecks that occur during serial dilutions, increasing the effective population size and potentially accelerating the rate of adaptive evolution.

However, chemostats also have drawbacks. They are much more costly to set up and difficult to operate. They are also more prone to contamination, and wall growth (in which cells stick to the chemostat vessel and thus avoid washout) negates the advantage of having a constant growth rate that is controlled by the dilution rate. By contrast, serial transfer is easy to perform precisely and without contamination. Also, there is no advantage to any cells that stick to the glassware, as they would be left behind at each serial transfer. Another important consideration is the replication that can be achieved, not only of the populations in the evolution experiment itself, but also the fitness assays that typically are performed under the same conditions. Two LTEE studies have run more than a thousand fitness assays (Wiser et al. 2013; Lenski et al. 2015), and others involved several tens or hundreds of assays. I cannot imagine running anywhere near that many using chemostats, but it has been feasible with the small-volume flasks and simple procedures employed in the LTEE.

Culture medium. Simplicity was an important consideration in designing the LTEE, and it was especially important when choosing the culture medium. We use Davis Mingioli (DM) medium with glucose at a concentration of 25 $\mu\text{g/mL}$, which we call DM25. Unlike many microbiological media, DM25 is a chemically defined medium. Thus, its composition is precisely known and can

be kept constant over time. Glucose is the limiting resource in terms of the density that the bacteria reach at stationary phase, with nitrogen and other required nutrients present in relative excess.

By using a chemically defined medium with a single, known limiting resource (as opposed to a rich, complex medium like LB), I sought to minimize the opportunities for stable coexistence of multiple lineages mediated by negative frequency-dependent selection (FDS). FDS occurs when the relative fitness of competing genotypes is not a constant, but instead depends on their relative abundance. Negative FDS means that each competitor is more fit when it is rare than when it is common, which facilitates coexistence by enabling invasion and preventing extinction. Relative fitness is a key metric for quantifying the repeatability and dynamics of evolution in the LTEE. With strong FDS, one would need multiple fitness metrics to account for different frequencies of the evolved and ancestral competitors. By minimizing FDS, we can usually employ a single fitness metric, which simplifies our analyses.

Population density. The glucose concentration in DM25 is low by microbiological standards. As a result, the bacteria reach a stationary-phase density of only $\sim 5 \times 10^7$ cells/mL. At that density, the cultures are only slightly turbid to the naked eye, and one cannot see any turbidity immediately after the dilution in fresh medium. With a culture volume of 10 mL, each population thus contains $\sim 5 \times 10^8$ cells at the end of the daily cycle. This small size might be expected to slow the rate of adaptive evolution in comparison to a larger population, at least if beneficial mutations are rare. However, I wanted a low population density to reduce the potential for metabolic byproducts to accumulate in the cultures. When cells grow, they may secrete byproducts that provide secondary resources or, in the case of toxic byproducts, inhibit growth. The presence of these byproducts can promote FDS.

As I said, I wanted to avoid frequency-dependent interactions because they would complicate interpretation of fitness measurements and trajectories. By having a low population density, the concentration of any byproducts should also be low, thereby reducing their ecological importance and minimizing the likelihood that complex interactions would evolve. Moreover, the fact that the populations are diluted 100-fold each day means that concentration of byproducts is even lower while the cells are growing exponentially. Nonetheless, we have discovered that some and even most LTEE populations show evidence of FDS, at least over portions of their evolutionary history (Rozen and Lenski 2000; Good et al. 2017). However, the frequency-dependent effects between genotypes within a population are typically small relative to the overall gains in fitness measured against the common ancestor (Elena and Lenski 1997; Maddamsetti et al. 2015). Therefore, we can usually ignore these complications when interpreting fitness trajectories measured over long periods of evolution.

With respect to the potential effect of a smaller population size on the rate of adaptation, I was not concerned because my previous experiment had shown that 400 generations was sufficient to see fitness gains. Indeed, I thought the smaller population size might even be helpful if the resulting dynamics reflected what is now called a strong-selection, weak-mutation regime (Gillespie 1991). In that case, it would be easier to detect selective sweeps from fitness trajectories alone, which might facilitate genetic analyses. As it turned out, however, beneficial mutations are abundant even at this population size (Wiser et al. 2013; Deatherage and Barrick 2021).

Why is citrate in the medium? Perhaps the most striking change that has occurred during the LTEE is that one population evolved the new ability to consume the citrate present in the DM25 medium (Blount et al. 2008). Even after 75,000 generations, none of the other populations has

evolved this capability. As a species, *E. coli* is characterized by its inability to grow on citrate in the presence of oxygen. Some *E. coli* strains can consume citrate under anoxic conditions, provided another energy source, such as glucose, is also available; however, the LTEE ancestor cannot do even that. Over the years, a few *E. coli* strains have been isolated from nature that are able to grow aerobically on citrate. These atypical strains evidently acquired that ability via horizontal transfer of plasmids from other species; however, there are no such opportunities in the LTEE.

Citrate is present in the DM medium as a chelating agent. Chelators bind metal ions. *E. coli* cells require trace amounts of iron for growth, and they can use ferric citrate to obtain that iron from the environment. The recipe for DM medium does not include iron. However, iron is generally present even in laboratory-grade water at concentrations that are sufficient to support growth, provided citrate is present. Years earlier, when I was a postdoctoral researcher, I had tried removing the citrate from the DM recipe, thinking it was an unnecessary complication. Without citrate, the cells grew well some days but failed to grow on others, perhaps reflecting fluctuations in water quality. In any case, I learned that citrate was an important component of the medium.

When I started the LTEE, I recall wondering whether the bacteria would evolve the ability to use the citrate in the medium. I imagined it might be easy—in which case all of them would gain that ability before too long—or it might prove impossible. As it turned out, the answer was more complicated and much more interesting. This paper is not the right venue to explain what has been learned to date about the evolution of the ability to consume citrate; that work is explained in depth elsewhere (Blount et al. 2012; Quandt et al. 2014; Quandt et al. 2015; Blount 2016; Bajić et al. 2018; Jagdish et al. 2020; Blount et al. 2020).

What about water quality? The culture medium is precisely controlled in all respects but one—namely, the quality of the water. Although we use laboratory-grade water when preparing culture medium, there are invariably minute traces of impurities. We also clean and inspect the glassware used in the LTEE with extra care, but inadvertent lapses in these procedures might also introduce impurities. However, any variation in the water quality appears to be inconsequential, at least in most respects. This inference is based on two lines of evidence. First, mean fitness trajectories are generally smooth and continuously increasing (Wiser et al. 2013; Lenski et al. 2015). Of course, there are occasional dips in the trajectories, but one should expect that given measurement noise (Lenski et al. 1991). If the water quality had changed meaningfully and systematically over time, such that the bacteria had adapted to distinct environments during different periods of the LTEE, then one would expect idiosyncratic rises and falls in the trajectories when fitness is measured in a later environment. Second, we explicitly tested whether fitness values are comparable across different laboratories when the LTEE moved from the University of California, Irvine, to Michigan State University, and again in anticipation of its move from Michigan State University to the University of Texas, Austin (Barrick et al. 2020).

We have observed one anomalous result that might reflect variation in the water quality. For over 10,000 generations, citrate-negative cells stably coexisted as a minority lineage in the population that evolved the ability to consume citrate (Blount et al. 2008; Turner et al. 2015). The citrate-negative lineage eventually went extinct, and we sought to understand the cause of that extinction by restarting 20 populations from each of two earlier timepoints. However, we were not able to replicate the extinction event in any of the replays. It is possible that variation in water quality caused the unexplained extinction, especially given the dual role of citrate as both a chelating agent and a consumable resource in that population. Alternatively, some other inadvertent perturbation

(for example, an error when making the culture media, or residual soap left after washing a flask) might have caused the extinction of the citrate-negative minority lineage.

Ancestral strain. The ancestor of the LTEE, which is called REL606, is a derivative of *E. coli* strain B (Daegelen et al. 2009; Studier et al. 2009). As with the culture medium, this choice reflected my prior experience with the B strain, going back to my postdoctoral work with Bruce Levin at the University of Massachusetts, Amherst, on the coevolution of bacteria and phages. Luria and Delbrück (1943) used *E. coli* B to demonstrate that mutations that confer resistance to phage happen randomly, before the cells encounter phage and hence before resistance confers a selective advantage. For decades, the B strain continued to serve as the workhorse for studies of bacteria-phage interactions and cell physiology. However, *E. coli* strain K-12 soon became an important alternative when Lederberg and Tatum (1946) demonstrated conjugation-mediated recombination, which eventually led to a detailed linkage map of the K-12 genome (Bachman and Low 1980). Strain K-12 and its derivatives are now more familiar to most microbiologists and geneticists; one of those derivatives is MG1655, which was the first *E. coli* genome to be fully sequenced (Blattner et al. 1997).

In the LTEE's early years, I recall some microbiologists questioning my choice of *E. coli* B, because they thought that K-12 would provide better opportunities for genetic analysis. That was a reasonable concern, but I was skeptical that we could make much progress on understanding the genetic basis of adaptation using traditional linkage mapping. My skepticism was based on the fact that mapping was a powerful tool for mutations that led to discrete changes in phenotypes—ones that could be screened by plating cells on specific media that would unambiguously distinguish “positive” and “negative” phenotypic states. I knew the LTEE would favor mutations that provided subtle improvements in growth in the glucose-limited DM25 medium, and I suspected they were unlikely to produce discrete losses or gains of function, which has been largely borne out (Cooper and Lenski 2000; Leiby and Marx 2014).

However, I did not have an alternative strategy in mind for identifying mutations responsible for fitness gains. We eventually made progress on the genetics based on two developments. First, I began collaborating with Dominique Schneider (Université Grenoble Alpes), a microbiologist with outstanding molecular and genetic skills, and our collaboration has continued for more than 20 years (Papadopoulos et al. 1999; Consuegra et al. 2021). Second, over time it became feasible and affordable to sequence genes (Lenski et al. 2003; Woods et al. 2006), then complete genomes (Barrick et al. 2009; Tenaillon et al. 2016), and eventually population metagenomes (Barrick and Lenski 2009; Good et al. 2017). Along the way, one student performed heroic experiments that used linkage mapping to identify a beneficial mutation (Stanek et al. 2009). To find a beneficial mutation in this way, we took advantage of previous work showing that mutations that increased fitness in the LTEE also typically made the cells larger, and it was faster to screen for cell volume than for fitness. Moreover, the *E. coli* B and K-12 linkage maps are largely syntenic for the genes they share, and their sequences are very similar for most core genes. Of course, the availability of inexpensive whole-genome sequencing means that one can now use any microbe of interest in an evolution experiment and make rapid progress on understanding the genetic basis of its adaptation (Burnetti and Ratcliff 2022). Also, the ability to both sequence and recombine genomes proved valuable for identifying a key mutation in the emergence of the unique citrate-consuming lineage (Quandt et al. 2014).

Another important benefit of using REL606 as the LTEE ancestor was the availability of a selectively neutral genetic marker that could be easily scored. During its derivation from the B

strain of Luria and Delbrück, a point mutation had been introduced by another researcher into a gene that rendered the cells unable to grow on the sugar L-arabinose (Studier et al. 2009). By plating a large number of cells onto an agar medium with arabinose as the sole carbon source, one can isolate reversion mutants. I isolated an Ara⁺ revertant before starting the LTEE, which I called REL607, and I confirmed that the Ara⁻ and Ara⁺ variants had equal fitness when they competed in the LTEE environment. The Ara marker serves two important purposes. First, we systematically alternate the Ara⁻ and Ara⁺ populations during the daily transfers. If we make a mistake in our sterile technique and advertently cross-contaminate one population with another, then we increase the likelihood that we will detect that problem when we periodically check the marker states. If such a problem is detected (or suspected), we can restart the impacted population from a previously frozen sample. In practice, this use of the Ara marker has become less important than it was in the early years of the LTEE, because all of the populations have evolved unique alleles at some loci; we can sequence those genes to determine whether a colony with a seemingly incorrect appearance is a contaminant or a mutant that arose within the correct population. Second, we use the marker in the competitive fitness assays. Specifically, we can compete evolved bacteria from an Ara⁻ population against the Ara⁺ ancestor, and vice versa, and then enumerate the competitors by plating them on an agar medium where they make colonies that can be distinguished by their colors.

The ability to compete the evolved and ancestral bacteria, and to tell them apart using a genetic marker, also presumes an absence of horizontal gene transfer (HGT). If HGT occurred, then the resulting recombination could dissociate the Ara marker from the fitness-relevant alleles that differ between the evolved and ancestral competitors. (Given the typically short duration of competition assays, HGT would likely not be a problem unless it occurred at a very high rate.) Many bacterial species, including *E. coli*, undergo HGT in nature (Reid et al. 2000; Tenaillon et al. 2010; Shapiro et al. 2012). However, the LTEE ancestor harbors no plasmids or functional prophages (viruses integrated into the chromosome) that could mediate HGT. Also, *E. coli* lacks the capacity for natural transformation via the uptake of extracellular DNA that some bacterial species possess. It would be fascinating if any of the LTEE populations somehow evolved HGT. However, we have seen no evidence of HGT-mediated recombination in genome sequences of clones from different lineages that have coexisted in some populations for many thousands of generations.

Absence of initial diversity. Each LTEE population was founded from a single haploid cell, thereby ensuring that its evolution depended entirely on new mutations (as opposed to pre-existing variation) and, moreover, the mutations were independent of mutations in other populations (as opposed to being identical by descent). That independence means that if we observe convergent phenotypic changes in replicate populations, those changes indicate parallel evolution, as opposed to collateral evolution that occurs when convergence arises from genetic variation that was present in a common ancestral population (Stern 2013; Lenski 2017a). In procedural terms, each LTEE population was started from a single cell by diluting a culture of the ancestral strain and spreading cells on an agar plate. Each colony that appeared on that plate was the outgrowth of a single cell. A different colony was then used to start each LTEE population. Of course, each colony contained millions of cells, so some mutations arose during the growth of the colony and hence occurred slightly before “time 0” in the LTEE. The key point, however, is that any and all mutations that spread through one evolving population arose independently of any and all mutations that spread through another population.

When I started the LTEE, the term “evolution experiment” was not widely used. While many previous experiments had studied the evolutionary process, they were usually called “selection

experiments” and most used fruit flies or other multicellular organisms. In fact, there was a long history of experimental evolution with microbes, but most evolutionary biologists were unfamiliar with that work (Mortlock 1984; Lenski 2017b). In any case, the replicate populations in selection experiments using multicellular organisms typically started from a base population that harbored substantial genetic variation. This initial variation was intentional, and indeed a point of emphasis, because it was essential in order to observe a response to selection over the timescale available to a typical study (Falconer 1960; Rose 1984). However, that shared initial variation also means that similar changes in replicate populations presumably result from collateral, and not truly parallel, evolution. That presumption is now supported by genomic analyses (Burke et al. 2010; Graves et al. 2017). Over deep time, however, mutations are the ultimate source of allelic variation, and the randomness of mutations is a critical feature of evolution. Hence, starting an experiment with shared genetic variation promotes collateral evolution and stacks the deck toward repeatable outcomes. The LTEE was designed to reflect the evolutionary process more fully, including the tension between the stochasticity of mutations and the determinism of selection across identical environments. Elsewhere in this issue, Burke (2023) discusses the important roles of both initial genetic variation and ongoing sexual recombination in evolution experiments with yeast.

Spontaneous mutation. The LTEE depends on spontaneous mutation to provide the genetic variation that fuels adaptation by natural selection. Most evolutionary biologists simply accept this approach, but some others with more applied interests have asked whether the bacteria should be mutagenized in order to accelerate their evolution. Although there might be some projects where increasing the mutation rate would be valuable, it is often unnecessary and can introduce problems. First, as explained above, I had run an experiment before starting the LTEE that showed we would see fitness gains within a reasonable timeframe. Second, the speed of adaptation in large asexual populations is not limited by the supply of new mutations owing to clonal interference among lineages that harbor competing beneficial alleles (Gerrish and Lenski 1998; de Visser et al. 1999; Wiser et al. 2013). Third, the fitness-impairing effects of deleterious mutations should become progressively more severe as one increases the genome-wide mutation rate. Fourth, any mutagenic procedure adds complexity to the experimental workflow. Moreover, one must consider potential safety and environmental issues with some mutagens. For projects where it is deemed important to increase the mutation rate, one should consider using strains with defects in DNA repair that cause hypermutability. Last, and perhaps most importantly for anyone designing a new experiment, increasing the mutation rate will increase the number of “passenger” mutations (i.e., neutral and weakly deleterious hitchhikers), which can make it more difficult to find the beneficial “driver” mutations that are responsible for adaptation. Gene-level parallelism provides a powerful signature for identifying beneficial mutations in the LTEE lineages that have retained the ancestral mutation rate, but that parallelism is obscured by widespread hitchhiking in those lines that evolved hypermutability (Tenaillon et al. 2016).

Acclimation and adaptation. A critical issue in any evolution experiment is distinguishing between strictly phenotypic acclimation and heritable adaptation. An advantage of working with *E. coli* and many other microbes is that they can be frozen and later revived, allowing a sort of “time travel” whereby organisms that lived at different times can be directly compared at the same time and in the same environment (Lenski and Travisano 1994). It is important to emphasize that when we analyze fitness or other properties of the LTEE populations, we do *not* directly compare the bacteria currently living in their flasks with earlier bacteria revived from the freezer. Instead, all the bacteria have been stored frozen. The samples of interest—for example, the ancestors and samples taken at later generations—are then revived in parallel. To ensure that all the samples are

comparably acclimated before measurements are made, the bacteria are grown for at least one full serial-transfer cycle under identical conditions. Those conditions are typically the same as during the LTEE proper, but there are exceptions. For example, we have measured fitness in different culture media (Travisano and Lenski 1996) and at different temperatures (Cooper et al. 2001) to characterize the bacteria's correlated responses to other environments. These correlated responses allow us to examine the specificity of the bacteria's adaptation to the LTEE environment.

Relative fitness. In the LTEE, we usually quantify adaptation by competing evolved samples against the ancestral strain under the same conditions as used in the LTEE. We use the ancestor with the opposite arabinose-marker state as the evolved bacteria to distinguish and enumerate the competitors. Also, we acclimate the competitors under those same conditions before starting the competition, as explained above. After an assay is finished and the competitors enumerated, we calculate the realized growth rates of the evolved and ancestral types during the head-to-head competition. We then express relative fitness as the ratio of the evolved bacteria's realized growth rate to that of the ancestor. This approach has at least two major advantages over measuring the growth rates of the ancestral and evolved bacteria separately. First, the competition assay integrates the effects of the same demographic phases that occur during the LTEE's daily transfer cycle: namely, the lag before population growth commences, exponential growth, later growth (including on any secreted metabolites), and death, if any, after the resources have been depleted. Second, any inadvertent deviations in the environmental conditions—an imperfectly cleaned flask or small temperature differences in an incubator—are experienced equally by the two competitors.

The question sometimes arises about whether it is appropriate to compete the evolved bacteria against the ancestor when measuring fitness trajectories. As discussed earlier, I chose to use the low-glucose minimal medium to minimize the potential for frequency-dependent effects. While negative FDS promotes coexistence of genotypes, other forms of FDS can lead to nontransitive competitive interactions. For example, in an experiment with yeast, Paquin and Adams (1983) reported an extreme case of non-transitivity in which an early evolved clone outcompeted and replaced the ancestral strain, a later evolved clone outcompeted and replaced the earlier evolved clone, but the later evolved clone was less fit than the ancestor in head-to-head competition. By contrast, de Visser and Lenski (2002) analyzed a series of competitions between 21 generational samples from one LTEE population and marked clones isolated at 4 intermediate timepoints from that population as well as the ancestor. They found that the fitness of evolved samples relative to one another was both qualitatively and quantitatively consistent with a transitive model of fitness effects. Nonetheless, as the bacteria's fitness has continued to increase over time in the LTEE, the statistical precision with which we can estimate further gains by competing the evolved bacteria against the ancestor declines. This limitation occurs because the evolved bacteria outcompete the ancestor to such an extent, even over a single transfer cycle, that the numbers of ancestral colonies on the plates used to enumerate the marked competitors become so low that they are subject to the heightened uncertainty associated with small numbers. To address this issue, we sometimes use competitors from intermediate generations to estimate more accurately the rates of ongoing fitness improvement (Wiser et al. 2013; Lenski et al. 2015).

Early in the LTEE's history, we showed that increased exponential growth rates and shortened lag phases accounted for almost all of the fitness gains measured during the first 2,000 generations (Vasi et al. 1994). Subsequent analyses indicate that changes in the exponential growth rate and the duration of the lag phase have remained the most important fitness components (Cooper et al. 2003; Atolia et al. 2020). These analyses of demographic parameters (with the measurements made

separately for the ancestral and evolved bacteria) also lend support to the idea that relative fitness in the LTEE is, to a first approximation, usually a simple matter of scramble competition for the limiting glucose.

Number of replicates. The LTEE has 12 replicate populations. I have been asked why I chose 12 replicates, as opposed to, say, 10 or 15. The simple reason was that the shaking incubators that we used (and still use) hold integer multiples of 12 of the small Erlenmeyer flasks that house the evolving populations. Moreover, we typically perform the competition assays to measure relative fitness for all 12 populations at the same time, so the incubator spaces used for those assays are also multiples of 12.

If I were to restart the LTEE today, it would be tempting to use 96-well microtiter plates with a robotic transfer system, such as used by Desai and collaborators in their evolution experiments with yeast (Lang et al. 2013; Johnson et al. 2021). That approach would allow greater replication of the independently evolving populations and the subsequent fitness assays. Even so, I would proceed carefully before going that route, based on problems we have sometimes encountered when using plastic microtiter plates for growth curves. At the least, I would consider using glass or glass-lined microtiter plates, and I would confirm that the plates can be adequately shaken during incubation to allow thorough mixing in each well and appropriate aeration without cross-contamination.

Project Management

Of course, the success of an experiment depends not only on its design, but also on its management and execution. Here I discuss two important issues that have arisen during the LTEE.

Freezer collection. We have frozen samples throughout the LTEE. Early in the experiment, we froze samples at 100-generation intervals (i.e., every 15 transfers), but later we switched to freezing samples every 500 generations. When making freezer stocks, we have always split the relevant culture between two vials in order to have a backup in case the primary vial is compromised or lost. (In recent years, we have split each sample between ~10 freezer vials, because we now get many requests to share samples with other labs.) We also have a rule within our lab that if someone plans to use a particular set of samples more than once as part of their research, they should regrow and refreeze the relevant samples to produce their own working stocks.

One thing I would change if I were to restart the LTEE is our numbering system to identify the frozen samples. The system we use is based on an old microbiological convention that strains should be identified by the researcher's initials followed by a number that reflects its order in that person's collection. However, there are two potential problems with that system. First, strains from other projects are interspersed with the LTEE samples. Second, the identifying number does not convey meaningful information about a sample, such as the source population and generation. Of course, we have recorded that information elsewhere, but not having that information on the actual freezer vials can lead to mistakes. As a case in point, we sent copies of the 75,000-generation samples to Jeff Barrick's lab at the University of Texas at Austin, where the LTEE is beginning its second scientific generation (Callaway 2022). When the new team checked the list of samples against the labels on the freezer vials, they saw that we had sent an incorrect sample because one digit was off. With hindsight, I think that an identifier like LTEE/Ara-6/75 (indicating LTEE population Ara-6 at generation 75,000) would be better than REL12244 for the whole-population samples that we periodically save.

The previous system might be retained for derivative samples, such as when someone isolates an individual clone from a whole-population sample, makes some genetic modification to a clone, or otherwise manipulates the material. It might also be worthwhile to have a unique identifier even for copies of the same sample, including especially copies made by subculturing an earlier sample. Some evolution may occur during subculturing procedures, especially in diverse whole-population samples, and unique identifiers might facilitate troubleshooting if it should ever become necessary. It is also helpful to have redundant labels on every sample container; for example, one could have a unique number on the top of each sample, with that number and a more meaningful description written on the side. In any case, I encourage anyone who is planning an evolution experiment to think carefully about freezer management.

We receive many requests each year for bacterial samples. Both the number and scope of these requests has increased over time, as the LTEE has become better known and new ways of studying bacteria have emerged. We honor all such requests, provided that the recipient scientist completes an institutionally mandated material transfer agreement, and that the requested samples have been mentioned in a publication. The latter point means that we would share samples through 60,000 generations, because we have published papers using those samples (Lenski et al. 2025; Good et al. 2017); however, we would not necessarily share the latest 75,000-generation samples, because we have not yet published anything about them. We typically ask the recipients to pay the shipping costs; we also reserve the right to have recipients pay for preparing the samples (e.g., in the event of a request for hundreds of samples), but we have never done so. I do not expect sample requests to lead to a collaboration or co-authorship on papers, although many collaborations have emerged from such requests. However, I do ask researchers who request samples about their interests and plans, for two reasons. First, I can help them identify the LTEE samples that are most relevant to their interests and plans. Second, I might know about similar work in progress, which helps avoid overlapping efforts. I have not always been successful in avoiding such overlap, but this approach has usually worked well.

Problems and restarts. Over the course of any long-running experiment, there will be mistakes and other interruptions. The frozen whole-population samples let us restart one or all of the LTEE populations from the last timepoint that samples were preserved. Doing so, however, introduces a perturbation, namely the freezing and thawing of samples. Moreover, before a sample is frozen, glycerol is added as a cryoprotectant; when the cells are thawed and returned to the standard DM25 medium, some of the glycerol is carried over and provides an additional resource for a day or two. Therefore, when a mistake occurs that is immediately recognized—for example, if a flask is knocked over during a transfer—we do not go back to the last frozen sample. Instead, each day after we have completed the transfers, we save the prior cultures in a refrigerator (rather than immediately discarding them). We can then use the refrigerated cultures to repeat the previous transfers. When this happens, we use the refrigerated cultures for all of the populations, not just the one impacted by the mistake, so that they all remain on the same schedule for the next time that we freeze samples. Using these refrigerated cultures also slightly perturbs the selective regime, but it is much less of a perturbation than freezing and thawing the bacteria.

There are also times when a problem is discovered only later—for example, a possible cross-contamination event. As noted earlier, we periodically check the arabinose-utilization phenotype, which we do by plating cells on an indicator agar medium where Ara⁻ and Ara⁺ cells make red and white colonies, respectively. Not all suspect colonies are contaminants, however, and nowadays we perform sequencing to determine whether a wrong-colored colony is a mutant or a contaminant

from another lineage. Interestingly, the suspect colonies we have seen in recent years have usually been false alarms—namely, Ara⁻ colonies in populations founded by the Ara⁺ ancestor that later evolved hypermutability, which would promote loss-of-function mutations in genes that affect that trait. In any case, when a cross-contaminant is discovered, we restart only the affected population from a frozen whole-population sample, and we time the restart such that all of the populations are on the same schedule for freezing future samples. As a consequence, some of the populations have run 500 generations, or multiples of 500 generations, behind the leading edge.

Alternative Designs

The experimental design of the LTEE has proven to be satisfactory, by and large, for the open-ended questions that it addresses. As I have emphasized, simplicity was a key consideration, and many other designs would have been more difficult to implement and, more so, to sustain for the decades that the LTEE has run. However, the design choices for the LTEE were certainly not the only reasonable ones, and it is interesting to wonder how the outcomes would differ had I made other choices. In fact, my lab has performed many other experiments using different designs to address more specific questions. These “descendant experiments” typically run for less than a year, although analyzing the resulting phenotypic and genetic changes takes much longer.

Izutsu et al. (2021) examined the effects of varying the dilution factor. They found that 100-fold dilutions are not only easy to perform, but reasonably close to the optimum for maximizing the rate of fitness increase, given the other parameters that influence the dynamics of adaptive evolution in the LTEE. De Visser et al. (1999) also varied the dilution factor along with the mutation rate of the founding strains, demonstrating that the rate of adaptation increases far more slowly than the mutation supply owing to clonal interference among lineages with competing beneficial mutations.

Two other descendant experiments examined the effects of plasmid-mediated HGT. Cooper (2007) showed that this process could accelerate adaptation in the context of the LTEE because it allowed beneficial mutations from competing lineages to recombine in a single genome and thus overcome clonal interference. By contrast, using *E. coli* K-12 strains as genetic donors and LTEE-derived B strains as recipients, Souza et al. (1997) observed no increase in the rate of fitness improvement compared to controls without HGT, even though later analyses showed that the HGT treatment had led to substantial introgression of K-12 sequences into the B genomes (Maddamsetti and Lenski 2018).

Other descendant experiments have investigated the effects of environments with different resources (Cooper and Lenski 2010; Satterwhite and Cooper 2015; Blount et al. 2020) and temperatures (Bennett and Lenski 1993; Mongold et al. 1996). Leroi et al. (1994) examined how phenotypic acclimation to different temperatures affected fitness measurements, and they showed that the acclimatory effects themselves could evolve.

Travisano et al. (1995) used diverged LTEE strains as ancestors to quantify the effects of history on adaptation to changed resource and temperature environments, while Card et al. (2019) examined the effect of history on adaptation to several antibiotics. Moore et al. (2000) examined the effects of deleterious mutations on subsequent adaptation using derivatives of an evolved LTEE strain that had undergone transposon mutagenesis, and they saw a strong tendency for compensatory evolution. Moore and Woods (2006) went much deeper into history by examining the evolution of different *E. coli* isolates derived from the wild in an environment similar to the

one used in the LTEE. Izutsu and Lenski (2022) studied the effects of initial diversity versus new mutations on the dynamics of adaptation to a new resource environment. Their starting populations ranged from individual clones to admixtures of whole populations derived from the LTEE. Although they observed strong selection among genotypes in the admixtures during the first 50 generations or so of their experiment, the average fitness of populations in the treatment that relied entirely on new mutations had caught up to the admixed populations by 500 generations.

In addition to their inherent scientific interest, these descendant experiments have often been an excellent way for graduate students and postdoctoral researchers to take ownership of a project. Indeed, many of these experiments grew out of questions that arose while those individuals were working on the LTEE itself. For example, the experiment on the joint effects of the mutation rate and dilution factor on the speed of adaptation (de Visser et al. 1999) followed from the discovery that some LTEE lines had evolved hypermutability (Sniegowski et al. 1997) and mathematical analyses of clonal interference (Gerrish and Lenski 1998). Moreover, descendant experiments can be easily moved and continued when individuals establish their own research labs.

Of course, one can imagine many alternative designs and possibilities for further experiments. Most evolution experiments are fairly inexpensive to run, so that time and imagination are often the limiting factors. Indeed, I think many microbiology labs that are *not* focused on evolutionary questions, but which have adequate resources and personnel, should consider running curiosity-driven evolution experiments as side projects. There is much to learn from watching organisms change and evolve over time, and new tools make it easier to generate and test hypotheses based on mutations that are readily discoverable by whole-genome sequencing (Martínez and Lang 2023).

Closing Thoughts

The core of evolutionary theory is conceptually simple, with a handful of processes that govern its dynamics: mutation and recombination produce heritable variation, while selection and drift determine the fate of variants. Yet when played out together over time and space in the natural world, these simple processes generate complex dynamics and interactions. Over the Earth's long history, those dynamics and interactions have, in turn, produced the amazing diversity of life—a “tangled bank” that is unique and difficult to disentangle. Paleontology and phylogenetic methods together have allowed evolutionary biologists to trace much of the history of life (Gould 1989; Dawkins 2004). Comparative studies, when cast in a phylogenetic framework, overcome some of the limitations associated with the unique history of life on Earth (Losos 2018). Intensive studies of evolution in action in natural populations allow fine-grained analyses of the dynamics of the evolutionary process (Grant and Grant 2014). Experimental evolution does not replace any of these other approaches; instead, it complements them by enabling in-depth, multifaceted analyses of evolutionary dynamics and outcomes under controlled settings with statistical replication.

Many evolution experiments seek to address one specific hypothesis in a targeted fashion, as experiments usually do in other fields. The LTEE, by contrast, explores several general and open-ended questions about the dynamics and repeatability of evolution. Its design was deliberately simple in order to minimize the opportunities for human error and, hopefully, to make the results easier to interpret. Although its design is simple, I think it is fair to say that the LTEE's results and interpretation have been rich, complex, and nuanced.

Levins (1966) addressed the inherent tension between simplicity and complexity in the context of building mathematical models. He wrote: “Thus population biology must deal simultaneously with genetic, physiological, and age heterogeneity within species of multispecies systems changing demographically and evolving under the fluctuating influences of other species in a heterogeneous environment. The problem is how to deal with such a complex system. The naive, brute force approach would be to set up a mathematical model which is a faithful, one-to-one reflection of this complexity.” Levins then went on to say: “Clearly we have to simplify the models in a way that preserves the essential features of the problem.” He then identified three desirable features of mathematical models, which he called generality, realism, and precision. He argued that most models sacrifice one of these goals to better fulfill the other two, with different modelers making different choices.

I think that Levins’ framework is useful for experimental evolution as well. The design of the LTEE favors generality and precision over realism. Of course, the specific phenotypic and genetic changes that occur in the LTEE populations are not generalizable, because these details invariably depend on the study organism and its environment. We have seen that clearly in some descendant experiments, where changes in the ancestors, temperature, and other factors influence the rate of fitness gain and its genetic underpinnings. However, the focal questions concerning the dynamics and repeatability of evolution, and the coupling of phenotypic and genomic changes, are of general interest to the field, and some of the overarching patterns we have documented may also prove to be broadly applicable. The LTEE’s simple environment allows precise measurements of relative fitness and the dynamics of adaptation. Also, using a well-studied model organism facilitates precise characterization and a deeper understanding of the molecular-genetic basis of the increased fitness and other phenotypic changes that we see. But without other species (including competitors and predators), diverse resources, spatial structure, and more, the environment lacks the complexity of nature. Hence, the LTEE lacks realism when compared to the complex environments in which organisms live and evolve in nature.

Over the years, several physicists have told me that I do biology like a physicist. In a similar vein, Levins suggested that “most physicists who enter population biology” also “sacrifice realism to generality and precision.” However, I am a biologist who seeks to understand the living world. Moreover, I fully appreciate that others might prioritize realism over generality, precision, or both when designing an evolution experiment, depending on their particular questions and hypotheses. For example, someone might want to understand a specific trait and its evolution in nature, which could require complex mesocosms (Scheinin et al. 2015) or, in the case of pathogens, propagation in hosts (Rodríguez-Pastor et al. 2022). In any case, the issues of generality, realism, and precision should be considered along with, and perhaps even before, designing an evolution experiment. In other words, “To thine own *goals* be true.”

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References

- Atolia E, Cesar S, Arjes HA, Rajendram M, Shi H, Knapp BD, Khare S, Aranda-Díaz A, Lenski RE, Huang KC (2020) Environmental and physiological factors affecting high-throughput measurements of bacterial growth. *mBio* 11:e01378-20
- Bachmann BJ, Low KB (1980) Linkage map of *Escherichia coli* K-12, edition 6. *Microbiol Rev* 44:1–56
- Bajić D, Vila JCC, Blount ZD, Sanchez A (2018) On the deformability of an empirical fitness landscape by microbial evolution. *Proc Natl Acad Sci USA* 115:11286–11291
- Barrick JE, Lenski RE (2009) Genome-wide mutational diversity in an evolving population of *Escherichia coli*. *Cold Spring Harbor Symp Quant Biol* 74:119–129
- Barrick JE, Yu DS, Yoon SH, Jeong H, Oh TK, Schneider D, Lenski RE, Kim JF (2009) Genome evolution and adaptation in a long-term experiment with *Escherichia coli*. *Nature* 461:1243–1247
- Barrick JE, Deatherage DE, Lenski RE (2020) A test of the repeatability of measurements of relative fitness in the long-term evolution experiment with *Escherichia coli*. In: Banzhaf W, Cheng BHC, Deb K, Holekamp KE, Lenski RE, Ofria C, Pennock RT, Punch WF, Whittaker DJ (eds) *Evolution in Action: Past, Present and Future*. Springer, Cham, Switzerland, pp 77–89
- Bennett AF, Lenski RE (1993) Evolutionary adaptation to temperature. II. Thermal niches of experimental lines of *Escherichia coli*. *Evolution* 47:1–12
- Blattner FR, Plunkett G, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* 277:1453–1462
- Blount ZD (2016) A case study in evolutionary contingency. *Studies Hist Phil Sci, Part C*:58:82–92
- Blount ZD, Borland CZ, Lenski RE (2008) Historical contingency and the evolution of a key innovation in an experimental population of *Escherichia coli*. *Proc Natl Acad Sci USA* 105:7899–7906
- Blount ZD, Barrick JE, Davidson CJ, Lenski RE (2012) Genomic analysis of a key innovation in an experimental *Escherichia coli* population. *Nature* 489:513–518
- Blount ZD, Maddamsetti R, Grant NA, Ahmed ST, Jagdish T, Baxter JA, Sommerfeld BA, Tillman A, Moore J, Slonczewski JL, Barrick JE, Lenski RE (2020) Genomic and phenotypic evolution of *Escherichia coli* in a novel citrate-only resource environment. *eLife* 9:e55414
- Burke MK (2023) Embracing complexity: yeast evolution experiments featuring standing genetic variation. *J Mol Evol*, this issue
- Burke MK, Dunham JP, Shahrestani P, Thornton KR, Rose MR, Long AD (2010) Genome-wide analysis of a long-term evolution experiment with *Drosophila*. *Nature* 467:587–590
- Burnetti A, Ratcliff WC (2022) Experimental evolution is not just for model organisms. *PLoS Biol* 20:e3001587
- Callaway E (2022) Legendary bacterial evolution experiment enters new era. *Nature* 606:634–635
- Card KJ, LaBar T, Gomez JB, Lenski RE (2019) Historical contingency in the evolution of antibiotic resistance after decades of relaxed selection. *PLoS Biology* 17:e3000397

- Consuegra J, Gaffé J, Lenski RE, Hindré T, Barrick JE, Tenaillon O, Schneider D (2021) IS-mediated mutations both promote and constrain evolvability during a long-term experiment with bacteria. *Nature Comm* 12:980
- Cooper TF (2007) Recombination speeds adaptation by reducing competition between beneficial mutations in populations of *Escherichia coli*. *PLoS Biol*:e225
- Cooper TF, Lenski RE (2010) Experimental evolution with *E. coli* in diverse resource environments. I. Fluctuating environments promote divergence of replicate populations. *BMC Evol Biol* 10:11
- Cooper TF, Rozen DE, Lenski RE (2003) Parallel changes in gene expression after 20,000 generations of evolution in *Escherichia coli*. *Proc Natl Acad Sci USA* 100:1072–1077
- Cooper VS, Lenski RE (2000) The population genetics of ecological specialization in evolving *Escherichia coli* populations. *Nature* 407:736–739
- Cooper VS, Bennett AF, Lenski RE (2001) Evolution of thermal dependence of growth rate of *Escherichia coli* populations during 20,000 generations in a constant environment. *Evolution* 55:889–896
- Daegelen P, Studier FW, Lenski RE, Cure S, Kim JF (2009) Tracing ancestors and relatives of *Escherichia coli* B, and the derivation of B strains REL606 and BL21(DE3). *J Mol Bio* 394:634–643
- Dawkins R (2004) *The Ancestor's Tale: A Pilgrimage to the Dawn of Evolution*. Houghton Mifflin, New York, NY
- Deatherage DE, Barrick JE (2021) High-throughput characterization of mutations in genes that drive clonal evolution using multiplex adaptome capture sequencing. *Cell Syst* 12:1187–1200.e4
- de Visser JAGM, Lenski RE (2002) Long-term experimental evolution in *Escherichia coli*. XI. Rejection of non-transitive interactions as cause of declining rate of adaptation. *BMC Evol Biol* 2:19
- de Visser JAGM, Zeyl CW, Gerrish PJ, Blanchard JL, Lenski RE (1999) Diminishing returns from mutation supply rate in asexual populations. *Science* 283:404–406
- Elena SF, Lenski RE (1997) Long-term experimental evolution in *Escherichia coli*. VII. Mechanisms maintaining genetic variability within populations. *Evolution* 51:1058–1067
- Falconer DS (1960) *Introduction to Quantitative Genetics*. Oliver & Boyd, Edinburgh, UK
- Fox JW, Lenski RE (2015) From here to eternity—the theory and practice of a really long experiment. *PLoS Biol* 13:e1002185
- Gerrish PJ, Lenski RE (1998) The fate of competing beneficial mutations in an asexual population. *Genetica* 102:127–144
- Gillespie JH (1991) *The Causes of Molecular Evolution*. Oxford Univ Press, Oxford, UK
- Good BH, McDonald MJ, Barrick JE, Lenski RE, Desai MM (2017) The dynamics of molecular evolution over 60,000 generations. *Nature* 551:45–50
- Gould SJ (1989) *Wonderful Life: The Burgess Shale and the Nature of History*. Norton, New York, NY
- Grant PR, Grant BR (2014) *40 Years of Evolution: Darwin's Finches on Daphne Major Island*. Princeton Univ Press, Princeton, NJ
- Graves JL Jr, Hertweck KL, Phillips MA, Han MV, Cabral LG, Barter TT, Greer LF, Burke MK, Mueller LD, Rose MR (2017) Genomics of parallel experimental evolution in *Drosophila*. *Mol Biol Evol* 34:831–842

- Izutsu M, Lenski RE (2022) Experimental test of the contributions of initial variation and new mutations to adaptive evolution in a novel environment. *Front Ecol Evol* 10:958406
- Izutsu M, Lake DM, Matson ZWD, Dodson JP, Lenski RE (2021) Effects of periodic bottlenecks on the dynamics of adaptive evolution in microbial populations. *bioRxiv* 2021.12.29.474457
- Jagdish T, Morris JJ, Wade BD, Blount ZD (2020) Probing the deep genetic basis of a novel trait in *Escherichia coli*. In: Banzhaf W, Cheng BHC, Deb K, Holekamp KE, Lenski RE, Ofria C, Pennock RT, Punch WF, Whittaker DJ (eds) *Evolution in Action: Past, Present and Future*. Springer, Cham, Switzerland, pp 107–122
- Johnson MS, Gopalakrishnan, S, Goyal J, Dillingham ME, Bakerlee CW, Humphrey PT, Jagdish T, Jerison ER, Kosheleva K, Lawrence KR, Min J, Moulana A, Phillips AM, Piper JC, Purkanti R, Rego-Costa A, McDonald MJ, Nguyen Ba AN, Desai MM (2021) Phenotypic and molecular evolution across 10,000 generations in laboratory budding yeast populations. *eLife* 10:e63910
- Lang GI, Rice DP, Hickman MJ, Sodergren E, Weinstock GM, Botstein D, Desai MM (2013) Pervasive genetic hitchhiking and clonal interference in forty evolving yeast populations. *Nature* 500:571–574
- Lederberg J, Tatum EL (1946) Gene recombination in *E. coli*. *Nature* 158:558
- Leiby N, Marx CJ (2014) Metabolic erosion primarily through mutation accumulation, and not tradeoffs, drives limited evolution of substrate specificity in *Escherichia coli*. *PLoS Biol* 12:e1001789
- Lenski RE (1988) Experimental studies of pleiotropy and epistasis in *Escherichia coli*. II. Compensation for maladaptive pleiotropic effects associated with resistance to virus T4. *Evolution* 42:433–440
- Lenski RE (2004) Phenotypic and genomic evolution during a 20,000-generation experiment with the bacterium *Escherichia coli*. *Plant Breeding Rev* 24:225–265
- Lenski RE (2017a) Convergence and divergence in a long-term experiment with bacteria. *Am Nat* 190:S57–S68
- Lenski RE (2017b) What is adaptation by natural selection? Perspectives of an experimental microbiologist. *PLoS Gen* 13:e1006668
- Lenski RE, Rose MR, Simpson SC, Tadler SC (1991) Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *Am Nat* 138:1315–1341
- Lenski RE, Travisano M (1994) Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. *Proc Natl Acad Sci USA* 91:6808–6814
- Lenski RE, Winkworth CL, Riley MA (2003) Rates of DNA sequence evolution in experimental populations of *Escherichia coli* during 20,000 generations. *J Mol Evol* 56:498–508
- Lenski RE, Wiser MJ, Ribeck N, Blount ZD, Nahum JR, Morris JJ, Zaman L, Turner CB, Wade BD, Maddamsetti R, Burmeister AR, Baird EJ, Bundy J, Grant NA, Card KJ, Rowles M, Weatherspoon K, Papoulis SE, Sullivan R, Clark C, Mulka JD, Hajela N (2015) Sustained fitness gains and variability in fitness trajectories in the long-term evolution experiment with *Escherichia coli*. *Proc R Soc Lond B* 282:20152292
- Leroi AM, Bennett AF, Lenski RE (1994) Temperature acclimation and competitive fitness: an experimental test of the beneficial acclimation assumption. *Proc Natl Acad Sci USA* 91:1917–1921
- Levins R (1966) The strategy of model building in population biology. *Am Sci* 54:421–431

- Losos JB (2018) *Improbable Destinies: Fate, Chance, and the Future of Evolution*. Penguin Random House, New York, NY
- Luria SE, Delbrück M (1943) Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* 28:491–511
- Maddamsetti R, Lenski RE (2018) Analysis of bacterial genomes from an evolution experiment with horizontal gene transfer shows that recombination can sometimes overwhelm selection. *PLoS Genetics* 14:e1007199
- Maddamsetti R, Lenski RE, Barrick JE (2015) Adaptation, clonal interference, and frequency-dependent interactions in a long-term evolution experiment with *Escherichia coli*. *Genetics* 200:619–631
- Martínez AA, Lang GI (2023) Identifying targets of selection in laboratory evolution experiments. *J Mol Evol*, this issue
- Mongold JA, Bennett AF, Lenski RE (1996) Evolutionary adaptation to temperature. IV. Adaptation of *Escherichia coli* at a niche boundary. *Evolution* 50:35–43
- Moore FB-G, Woods R (2006) Tempo and constraint of adaptive evolution in *Escherichia coli* (Enterobacteriaceae, Enterobacteriales). *Biol J Linn Soc* 88:403–411
- Moore FB-G, Rozen DE, Lenski RE (2000) Pervasive compensatory adaptation in *Escherichia coli*. *Proc R Soc B* 267:515–522
- Mortlock RP, editor (1984) *Microorganisms as Model Systems for Studying Evolution*. Plenum, New York, NY
- Papadopoulos D, Schneider D, Meier-Eiss J, Arber W, Lenski RE, Blot M (1999) Genomic evolution during a 10,000-generation experiment with bacteria. *Proc Natl Acad Sci USA* 96:3807–3812
- Paquin CE, Adams J (1983) Relative fitness can decrease in evolving asexual populations of *S. cerevisiae*. *Nature* 306:368–371
- Quandt EM, Deatherage DE, Ellington AD, Georgiou G, Barrick JE (2014) Recursive genomewide recombination and sequencing reveals a key refinement step in the evolution of a metabolic innovation in *Escherichia coli*. *Proc Natl Acad Sci USA* 111:2217–2222
- Quandt EM, Gollihar J, Blount ZD, Ellington AD, Georgiou G, Barrick JE (2015) Fine-tuning citrate synthase flux potentiates and refines metabolic innovation in the Lenski evolution experiment. *eLife* 4:e09696
- Reid SD, Herbelin CJ, Bumbaugh AC, Selander RK, Whittam TS (2000) Parallel evolution of virulence in pathogenic *Escherichia coli*. *Nature* 406:64–67
- Rodríguez-Pastor R, Shafran Y, Knossow N, Gutiérrez R, Harrus S, Zaman L, Lenski RE, Barrick JE, Hawlena H (2022) A roadmap for in vivo evolution experiments with blood-borne parasitic microbes. *Mol Ecol Res* 22:2843–2859
- Rose MR (1984) Laboratory evolution of postponed senescence in *Drosophila melanogaster*. *Evolution* 38:1004–1010
- Rozen DE, Lenski RE (2000) Long-term experimental evolution in *Escherichia coli*. VIII. Dynamics of a balanced polymorphism. *Am Nat* 155:24–35
- Satterwhite RS, Cooper TF (2015) Constraints on adaptation of *Escherichia coli* to mixed-resource environments increase over time. *Evolution* 69: 2067–2078
- Scheinin M, Riebesell U, Rynearson TA, Lohbeck KT, Collins S (2015). Experimental evolution gone wild. *J Roy Soc Interface* 12:20150056

- Shapiro BJ, Friedman J, Cordero OX, Preheim SP, Timberlake SC, Szabó G, Polz MF, Alm EJ (2012) Population genomics of early events in the ecological differentiation of bacteria. *Science* 336:48–51
- Sniegowski PD, Gerrish PJ, Lenski RE (1997) Evolution of high mutation rates in experimental populations of *Escherichia coli*. *Nature* 387:703–705
- Souza V, Turner PE, Lenski RE (1997) Long-term experimental evolution in *Escherichia coli*. V. Effects of recombination with immigrant genotypes on the rate of bacterial evolution. *J Evol Biol* 10:743–769
- Stanek MT, Cooper TF, Lenski RE (2009) Identification and dynamics of a beneficial mutation in a long-term evolution experiment with *Escherichia coli*. *BMC Evol Biol* 9:302
- Stern DL (2013) The genetic causes of convergent evolution. *Nature Rev Gen* 14:751–764
- Studier FW, Daegelen P, Lenski RE, Maslov S, Kim JF (2009) Understanding the differences between genome sequences of *Escherichia coli* B strains REL606 and BL21(DE3) and comparison of the *E. coli* B and K-12 genomes. *J Mol Biol* 394:653–680
- Tenaillon O, Skurnik D, Picard B, Denamur E (2010) The population genetics of commensal *Escherichia coli*. *Nature Rev Microbiol* 8:207–217
- Tenaillon O, Barrick JE, Ribeck N, Deatherage DE, Blanchard JL, Dasgupta A, Wu GC, Wielgoss S, Cruveiller S, Médigue C, Schneider D, Lenski RE (2016) Tempo and mode of genome evolution in a 50,000-generation experiment. *Nature* 536:165–170
- Travisano M, Lenski RE (1996) Long-term experimental evolution in *Escherichia coli*. IV. Targets of selection and the specificity of adaptation. *Genetics* 143:15–26
- Travisano M, Mongold JA, Bennett AF, Lenski RE (1995) Experimental tests of the roles of adaptation, chance, and history in evolution. *Science* 267:87–90
- Turner CB, Blount ZD, Lenski RE (2015) Replaying evolution to test the cause of extinction of one ecotype in an experimentally evolved population. *PLoS ONE* 10:e0142050
- Vasi F, Travisano M, Lenski RE (1994) Long-term experimental evolution in *Escherichia coli*. II. Changes in life-history traits during adaptation to a seasonal environment. *Am Nat* 144:432–456
- Wiser MJ, Ribeck N, Lenski RE (2013) Long-term dynamics of adaptation in asexual populations. *Science* 342: 1364–1367
- Woods R, Schneider D, Winkworth CL, Riley MA, Lenski RE (2006) Tests of parallel molecular evolution in a long-term experiment with *Escherichia coli*. *Proc Natl Acad Sci USA* 103:9107–9112