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WINOGRADSKY REVIEW

Experimental evolution and the dynamics of adaptation and genome evolution in microbial populations

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Evolution is an on-going process, and it can be studied experimentally in organisms with rapid generations. My team has maintained 12 populations of *Escherichia coli* in a simple laboratory environment for >25 years and 60 000 generations. We have quantified the dynamics of adaptation by natural selection, seen some of the populations diverge into stably coexisting ecotypes, described changes in the bacteria's mutation rate, observed the new ability to exploit a previously untapped carbon source, characterized the dynamics of genome evolution and used parallel evolution to identify the genetic targets of selection. I discuss what the future might hold for this particular experiment, briefly highlight some other microbial evolution experiments and suggest how the fields of experimental evolution and microbial ecology might intersect going forward.
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> The distinction between 'ecological time' and 'evolutionary time' is artificial and misleading. Changes of both kinds may be on any time scale: frequently genetic and ecological changes are simultaneous—Janis Antonovics (1976).

Introduction

Evolution is often viewed as an inexorably slow process, one that happens over extremely long periods. By contrast, ecology is typically seen as occurring much faster, reflecting both the intrinsic dynamics of biotic interactions and the increasingly pervasive effects of anthropogenic environmental change. In fact, however, evolution is simply the conjunction of ecology and genetics, which involve a few basic processes—births, deaths, competition, mutation and selection—that have been happening since life began and will continue for as long as life exists.

On-going evolution has been studied in such organisms as finches in the Galapagos archipelago (Grant and Grant, 2014) and fruit flies in the laboratory (Burke *et al.*, 2010). But nowhere is evolution in action more apparent than in microorganisms. Examples include such striking and impactful changes as antibiotic resistance in bacteria and altered host-range in viruses, as well as subtle changes that allow the spread of pathogens to be tracked by epidemiologists or that produce microbes slightly better adapted to their environments than their predecessors.

In this review, I will focus mostly on an experimental study in which a single bacterial species has been evolving under constant conditions for many years. Such 'pure culture' studies have sometimes been criticized because they eliminate the richness and complexity of the natural world. I cannot dispute that point. However, science often progresses by simplifying nature in order to probe and make sense of more tractable systems. Moreover, one of the most striking outcomes of the experiment has been the richness and complexity that have emerged.

The long-term evolution experiment

Structure of the experiment and motivating questions

The long-term evolution experiment, or LTEE, is simple both conceptually and practically. Twelve populations were started the same ancestral strain of *Escherichia coli* in 1988. The ancestral strain has no plasmids or functional prophages, and *E. coli* is not naturally transformable, so there is no horizontal gene transfer. However, each population has

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millions of cells that provide a continual supply of new mutations. The populations are propagated in a glucose-limited minimal salts medium at 37 °C by transferring 1% of the volume into fresh medium every day. The 100-fold dilution and resulting regrowth allows $\log_2 100 \approx 6.7$ generations each day. Samples from each population are periodically stored at −80 °C, where they are available for later study. Importantly, the frozen cells remain viable, such that changes in performance can be analyzed at later times; and when accidents occur, the populations are restarted from the most recent whole-population samples. As of this writing, the LTEE has passed 66 000 generations.

Since its inception, the LTEE has examined several overarching questions (Lenski *et al.*, 1991). Is the process of adaptation by natural selection invariably slow and gradual, or are there periods of rapid change and stasis? Does fitness eventually reach some maximum level, or can organisms continue to improve indefinitely, even in a constant environment? Will the replicate populations achieve the same fitness peaks, or will some discover better solutions than others? If fitness trajectories evolve in parallel, does that imply the same underlying genetic changes? How are phenotypic and genetic evolution coupled, both dynamically and functionally?

Dynamics of adaptation by natural selection

We can directly measure the extent of adaption by natural selection by competing bacteria from a later-generation sample against the ancestral strain. Relative fitness is expressed as the ratio of the realized growth rates of the evolved and ancestral bacteria as they compete with one another. Unless otherwise stated, the competitions are performed in the same medium and conditions as the LTEE; however, we can also vary the conditions to examine how that affects performance. The competitions usually begin with an equal mix of ancestral and evolved bacteria, but we sometimes vary the starting ratio to look for more complex frequency-dependent interactions. Of course, we must be able to distinguish the ancestral and evolved bacteria to enumerate them and calculate their realized growth rates. To that end, we use two variants of the ancestral strain that differ by a genetic marker that allows us to distinguish the competitors; the marker is neutral (that is, does not affect fitness) under the conditions of the LTEE. Before the ancestral and evolved competitors are mixed, both have been frozen, thawed and grown separately for a full transfer cycle under the conditions where they will compete. This acclimation ensures that any significant differences in competitive fitness reflect genetic differences rather than non-heritable differences in physiological state.

Looking at the fitness trajectory for one population over the first 2000 generations (Lenski and Travisano, 1994), we see that fitness increased by ~30% (Figure 1a). The rise appears to involve three discrete steps of ~10% each. This pattern closely matches the dynamic one expects in a large, initially homogeneous asexual population. Consider a mutation that confers a 10% benefit. Assuming it is not lost to random genetic drift while it is rare, then it takes ~250 generations for that mutation to become the majority. For most of that time the mutant remains a tiny minority because, assuming a constant growth-rate differential, the ratio of the mutant to its progenitor changes exponentially. Therefore, the fitness trajectory, at least in the early generations, is dominated by sequential selective sweeps of a few mutations with large beneficial effects. Other beneficial mutations occur as well, but they are outcompeted by the most beneficial ones, a phenomenon called clonal interference (Gerrish and Lenski, 1998; Maddamsetti *et al.*, 2015). When clones from this population were sequenced, five mutations were found and each mutation was shown to be beneficial (Khan *et al.*, 2011). The fact that there were five beneficial mutations, despite only three step-like increases in fitness, indicates that some sweeps involved multiple beneficial mutations (Lang *et al.*, 2013; Maddamsetti *et al.*, 2015).

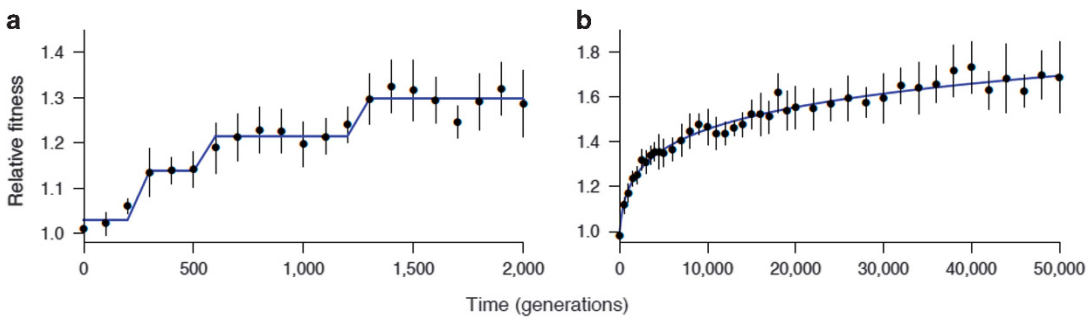

Figure 1 Fitness trajectories of evolving *E. coli* populations. (**a**) Fitness trajectory for one population, Ara–1, relative to its ancestor over the first 2000 generations of the LTEE. Error bars are 95% confidence intervals based on replicated assays. The line segments show the fit of a step model to the data. Modified from Lenski and Travisano (1994). (**b**) Trajectory for the grand-mean fitness across the LTEE populations over 50 000 generations. Error bars are 95% confidence limits based on replicate populations. The curve shows the fit of a power-law model. Modified from Wiser *et al.* (2013).

We have also characterized the grand-mean fitness trajectory across the replicate populations and 50 000 generations (Wiser *et al.*, 2013). Over this timeframe, the bacteria reached a relative fitness of ~1.7, meaning they grew ~70% faster than their ancestor when competing in the LTEE environment (Figure 1b). Their rate of improvement slows over time, as it becomes more difficult to achieve further gains after taking the low-hanging fruit. Does this slowdown imply that the bacteria have, or eventually will, hit some limit on their fitness? Wiser *et al.* (2013) compared the fit of two simple models with these data, each of which has two free parameters. One model, a rectangular hyperbola, has an asymptote. The other model, a power-law, has no upper bound, but the rate of increase declines with the logarithm of time. Both models fit the data well, but the power-law model fits the data better. Moreover, the power-law relation is much better at predicting the future than the hyperbolic model. When a truncated data set was used to predict the future trajectory, the hyperbolic model systematically underestimated the potential for further fitness gains. By contrast, the power-law model predicted with impressive accuracy the improvement out to 50 000 generations using only 5000 generations of data (Wiser *et al.*, 2013).

The power-law model has no upper bound, and so one might reasonably worry that it predicts the bacteria will eventually grow at a rate that is biophysically implausible. However, it does not predict implausible growth rates for the foreseeable future because the rate of improvement scales with the logarithm of time. The ancestral strain used to found the populations had a doubling time of ~55 min in the glucose-limited minimal medium of the LTEE (Vasi *et al.*, 1994). Wiser *et al.* (2013) extrapolated the model to 2.5 billion generations, which corresponds to 50 000 generations of scientists running the LTEE for 50 000 generations each. At that point, the projected doubling time is ~23 min. That value would be impressive for bacteria growing in a minimal medium, but it is no faster than many *E. coli* strains can grow in nutrient-rich media, and some species can grow even faster. Thus, the power-law model generates plausible predictions far into the future.

One might also ask whether it is appropriate to base inferences about the dynamics of adaptation on competitions against an ancestor that is increasingly distant in time. This approach implicitly assumes that relative fitnesses are transitive; for example, if strain B is 10% more fit than strain A, and strain C is 10% more fit than B, then we expect C to be 21% more fit than A when relative fitnesses are multiplicative. In most natural circumstances, this assumption would be unreasonable over long periods, and it would not be met in many laboratory systems with complex ecological feedbacks mediated by predators, secreted metabolites and the like. However, this assumption appears to be valid in the LTEE, at least to a first approximation and in most cases, based on assays involving competitors from different generations (de Visser and Lenski, 2002). The extreme simplicity of the LTEE environment undoubtedly accounts for the transitivity; in particular, the low concentration of glucose limits the population density, which in turn limits the accumulation of by-products in the medium. Nonetheless, as discussed below, subtle frequency-dependent interactions involving by-products have arisen in some populations, but they have fitness effects that are small in comparison with the overall gains in fitness relative to the ancestor. In any case, to the extent that fitness trajectories based on competition against the ancestor are inaccurate, they should generally underestimate the extent of continuing adaptation (Wiser *et al.*, 2013). That is because genotypes that systematically increase in frequency in large populations must be more fit than those they replace, but that need not be the case relative to some type that is no longer present (Paquin and Adams, 1983).

Evolving complexity

The LTEE was designed to be simple. Having just one species, one limiting resource and no spatial structure should minimize complex ecological interactions, and the absence of horizontal gene transfer was meant to simplify genetic analyses. Nonetheless, unexpected and interesting complexities arose spontaneously as a consequence of the bacteria evolving within these narrow confines.

On the ecological front, one population, called Ara–2, has diverged into two distinct lineages, called L and S, which have coexisted for over 50 000 generations. There are at least two ways that coexistence could occur, even in this simple environment. First, one ecotype might grow faster at the concentration of glucose available at the start of each day, whereas the other type may grow relatively faster as the concentration is drawn down. Second, one type might be the superior competitor for the glucose, whereas the other better uses some secreted by-product, generating a cross-feeding interaction. In population Ara–2, coexistence depends primarily on the second mechanism. The L ecotype grows faster on glucose, but it cannot grow in its own spent medium, which the S type is able to use (Rozen and Lenski, 2000). It appears that acetate is the main by-product that supports the S lineage (Großkopf *et al.*, 2016). During the establishment of this polymorphism, the L ecotype lost its ability to use acetate whereas the S type improved that ability. As a result of the trade-off between growth on glucose and acetate, the two ecotypes can stably coexist. That is, each type has a competitive advantage when rare, but as its frequency increases, it loses its advantage. However, evolution has continued in both lineages (Le Gac *et al.*, 2012). As one or the other acquired new beneficial mutations, they perturbed the

relationship, leading to large fluctuations in their relative abundance over longer periods (Rozen and Lenski, 2000). In principle, one ecotype might evolve in such a way that it drives the other extinct, but that has not yet happened in population Ara–2.

Other populations generated more transient polymorphisms (Maddamsetti *et al.*, 2015). In population Ara–1, two lineages coexisted from ~7000 to ~14 000 generations, before one drove the other extinct (Figure 2). It is not known why the polymorphism in Ara–2 has persisted longer than those in other populations, but a likely explanation is that the L ecotype lost its ability to use acetate. In other populations, the ecotype that is the better competitor for glucose might retain its ability to use acetate. If so, the advantage to a minority ecotype with better growth on acetate would be less, and a smaller improvement in the dominant type might be sufficient to drive the minority type to extinction.

On the genetics front, complexity arose via changes in the mutation rate (Sniegowski *et al.*, 1997; Barrick *et al.*, 2009; Blount *et al.*, 2012; Tenaillon *et al.*, 2016). Six populations evolved hypermutability caused by mutations affecting either DNA mismatch repair or the ability to remove certain oxidized bases. The changes are not subtle, with increases in the point mutation rate of ~100-fold, although several populations later evolved reduced hypermutability (Figure 3). The hypermutability affects not only the mutation rate but also the spectrum of base substitutions. In addition to the populations that evolved higher point mutation rates, population Ara+1 evolved increased activity of a native transposable element, IS*150*, which led to increased rates of insertion mutations as well as chromosomal rearrangements (Papadopoulos *et al.*, 1999; Raeside *et al.*, 2014; Tenaillon *et al.*, 2016). These changes complicate genetic analyses. In particular, the increased mutation rates make it more difficult to identify the 'driver' mutations that improve fitness because they are surrounded by many more 'passenger' mutations that are neutral or even deleterious. (Nevertheless, by focusing on populations that did not evolve hypermutability, and other lineages before they became hypermutable, we can identify genetic targets of selection in the LTEE. I will return to that topic in a later section).

The emergence of hypermutators raises the questions of how and why they evolve. At first glance, they might seem to evolve because their higher mutation rate speeds the process of adaptation by natural selection. That explanation is correct, but also incomplete. Beneficial mutations are only a small fraction of all mutations, and a random mutation is more likely to be deleterious than beneficial. From that perspective, a bacterium with an increased mutation rate has a disadvantage because its progeny tend to be less fit. However, natural selection depends not only on average fitness

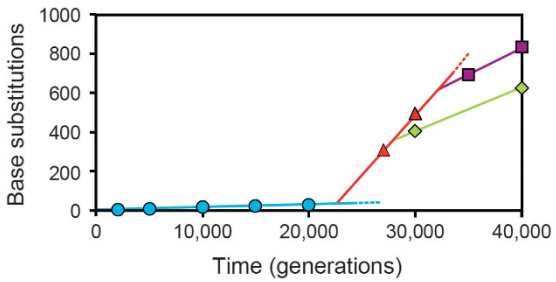

Figure 3 Changing mutation rate in population Ara–1. This population maintained the low ancestral mutation rate for over 20 000 generations. It then evolved hypermutability that increased its point mutation rate, with partial compensation later occurring in parallel in two lineages within that population. Modified from Wielgoss *et al.* (2013).

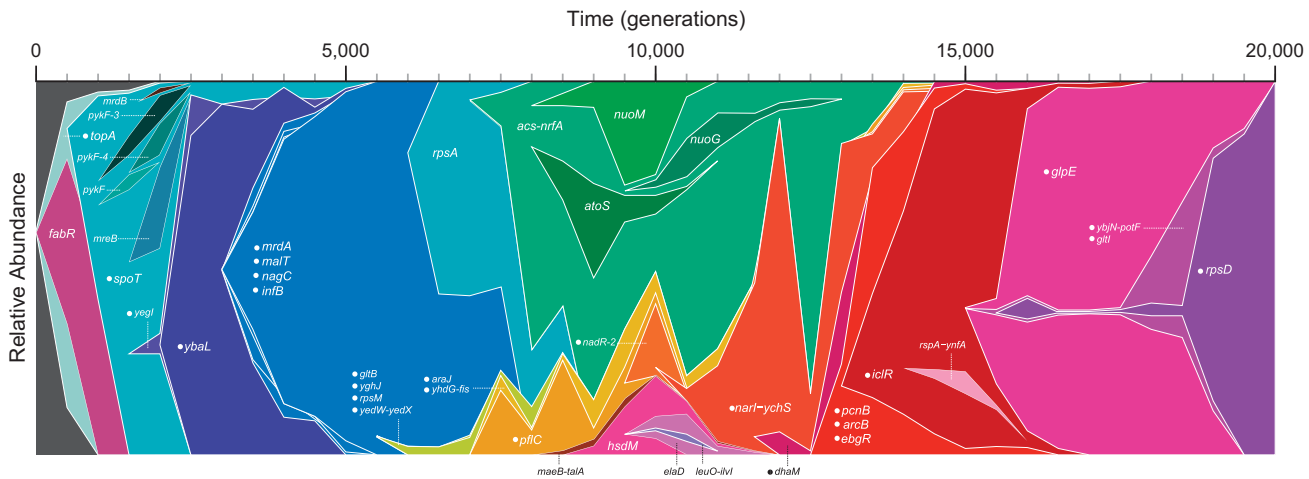

Figure 2 Muller plot showing the relative abundances of 42 mutations found in population Ara–1 during its first 20 000 generations. The labels are names of the mutated genes; dots before the gene name indicate mutations that eventually fixed in the population. Note the period between ~7000 and ~14 000 generations, when two major clades—one shown in shades of green, and the other in yellows and reds—transiently coexisted before the latter eventually drove the former extinct. Coexistence was supported by a negative frequency-dependent interaction, with the fluctuations evidently caused by beneficial mutations that gave one lineage or the other a temporary advantage. Modified from Maddamsetti *et al.* (2015).

effects, but also on the variance in fitness, and a hypermutable lineage may prevail if it produces the next big winner. In an asexual population, the allele that causes hypermutability is inherited along with any beneficial mutation that a hypermutator happens to produce, generating indirect selection for hypermutability (Tenaillon *et al*., 2001). But if an allele that causes hypermutability is usually at a disadvantage, how does it become common enough to generate the rare winner? In fact, most mutations that cause hypermutability will never produce a winner; instead, they will go extinct. However, given recurrent mutations in the genes that encode the proteins that perform mismatch repair and remove mutagenic compounds, occasionally such a mutant will get lucky before it goes extinct and produce a beneficial mutation that more than offsets its harmful effect, allowing the hypermutator to increase in frequency. The probability that this happens depends on the opportunity for beneficial mutations. As a general tendency, the further a population is from its optimum, the more scope there is for improvement and the greater the opportunity for beneficial mutations (Moore *et al*., 2000; Wilke *et al*., 2003; Kryazhimskiy *et al*., 2014). Consequently, when a population encounters a new environment—such as at the start of the LTEE—there are many opportunities for hypermutators to generate beneficial mutations and become established. Then, as the population becomes better adapted to those conditions, the opportunity for further improvement is reduced while the cost of deleterious mutations continues unabated. As a result, a lower mutation rate may evolve once the fitness gain that results from reducing the load of deleterious mutations becomes greater than the gains associated with beneficial mutations that the hypermutator can generate (Figure 3). In short, the mutation rate is subject to an evolutionary tension between opposing effects (Wielgoss *et al*., 2013; Good and Desai, 2016).

Innovation and contingency

The LTEE populations live in a medium with glucose as the limiting resource. Citrate is also present as a chelating agent, but most *E. coli* cannot transport citrate into the cell when oxygen is present. The inability to grow aerobically on citrate is one of the defining features of *E. coli* as a species. Some strains can consume citrate under anoxic conditions, although doing so involves co-metabolism and requires another source of carbon and energy. After ~31 000 generations, however, bacteria in population Ara–3 gained the ability to consume citrate in the oxygen-rich LTEE environment (Blount *et al*., 2008). Moreover, these Cit$^+$ cells can grow on citrate as a sole carbon source. This population was not hypermutable when this new trait arose, although it later evolved hypermutability. None of the other populations has evolved this ability even after >65 000 generations. Given the population size, number of generations, and ancestral mutation rate, every point mutation has been tested many times over in each population. Why, then, was it so difficult to evolve the Cit$^+$ phenotype?

One possibility is that the transition from Cit$^-$ to Cit$^+$ required multiple mutations, so that the right genetic background had to be in place, after which (and only after which) another mutation would yield the new function. A second possibility is that some rare type of mutation was necessary to produce the Cit$^+$ phenotype. These hypotheses are not mutually exclusive; in fact, both factors contributed to the difficulty of this transition under the conditions of the LTEE. To examine the effect of genetic background, Blount *et al.* (2008) performed 'replay' experiments in which they attempted to evolve the Cit$^+$ phenotype starting from the ancestor and Cit$^-$ clones isolated at various times. In light of the difficulty of generating Cit$^+$ mutants, most replays involved plating large numbers of cells on an agar-based medium that contained citrate but no glucose, incubating the plates for several weeks, and seeing whether any Cit$^+$ mutants emerged. Although Cit$^+$ mutants were very rare, the replays showed that genetic context mattered: neither the ancestor nor any clone that had been isolated before generation 20 000 produced any Cit$^+$ mutants, but 17 mutants arose from later clones. Thus, the origin of this new function was historically contingent; that is, the propensity to evolve the Cit$^+$ phenotype depended on one or more previous changes.

Whole-genome sequencing was performed to identify the mutations responsible for the new function (Blount *et al*., 2012). The 'actualizing' mutation that first produced a Cit$^+$ cell was found to be a tandem duplication involving a gene, *citT*, which encodes a protein used to transport citrate in the absence of oxygen. The effect of the duplication was that the second copy of the gene was adjacent to a new upstream regulatory region, which was downstream of the original copy. As a consequence of this 'promoter capture' event, the *citT* gene was expressed in the presence of oxygen as glucose ran out, allowing the cells to switch to growing on citrate. All of the Cit$^+$ mutants from the replay experiments also had rearrangement mutations that involve the *citT* gene and appear to enable expression from alternative promoters. Evidently, no point mutation can generate growth on citrate, even in an otherwise suitable genetic background. Moreover, the relevant rearrangements must happen at very low rates under the specific conditions of the LTEE. The duplicated region then underwent further amplification in population Ara–3, which increased its growth on citrate (Blount *et al*., 2012).

To see how genetic context affected citrate metabolism, the relevant module—the *citT* gene and its newly captured regulatory region—were moved onto a high-copy plasmid, which was then transformed into four clonal backgrounds (Blount *et al*., 2012). Three of the transformed clones,

including the ancestral strain, could grow on citrate in the presence of oxygen, albeit with long delays between the depletion of glucose and the start of growth on citrate and other difficulties (Figure 4a). By contrast, the transformant of a Cit$^-$ clone that was closely related to the Cit$^+$ lineage in population Ara–3 made a seamless transition from glucose to citrate and reached a high density (Figure 4b). These results indicate epistasis between the citrate-transport module and genetic background. More recent studies have identified chromosomal mutations that, along with that module, enable vigorous growth on citrate (Quandt *et al.*, 2014, 2015).

As interesting as this function is from a genetics perspective, it is equally interesting from an ecological standpoint. When the Cit$^+$ type evolved, it did not drive the Cit$^-$ bacteria extinct, nor did the Cit$^+$ bacteria lose their ability to grow on glucose; instead, the two types coexisted for over 10 000 generations before the Cit$^-$ lineage went extinct (Turner *et al.*, 2015). Coexistence was possible, in part, because the Cit$^+$ bacteria had a longer lag before growth than the Cit$^-$ bacteria when they were transferred into fresh medium (Blount *et al.*, 2008). The Cit$^+$ cells grew on citrate before entering stationary phase, so they probably had to change their metabolic state to a greater extent than did the Cit$^-$ cells in order to grow on glucose. Also, the evolution of Cit$^+$ bacteria led to even more complex ecological interactions as a consequence of the biochemical mechanism they use to obtain citrate. The *citT* gene encodes an antiporter protein that, while bringing a citrate molecule into the cell, exports a succinate, fumarate or malate molecule to the medium. Citrate is energetically more valuable than these compounds, but *E. coli* can nonetheless use them for growth. In fact, both the Cit$^-$ and Cit$^+$ lineages evolved improved growth on succinate (Turner, 2015). Thus, the interactions in this flask ecosystem became increasingly complex as a consequence of evolution, with competition for not one, not two, but several potential sources of carbon and energy.

Genome dynamics

How many mutations have occurred in the LTEE? How have they accumulated over time? In answering these questions, it is critically important to distinguish between the number of mutational events that have occurred and the number of mutational differences between an evolved genome and its ancestor. What follows is a rough approximation for the former in a nonhypermutable population through 50 000 generations. The generations took 7500 days of 100-fold dilution and regrowth; each regrowth produced ~ 3×10^8 cells; each cell's genome has ~ 5×10^6 bp; and the point mutation rate is ~ 10^{-10} per bp per cell replication. The resulting product is over one billion for point mutations alone, and even more with insertions, deletions and other types of mutations. However, the vast majority of mutations are lost by random genetic drift during the daily dilutions; many others are eliminated by natural selection because they have deleterious effects; even most beneficial mutations are lost by drift or outcompeted by other genotypes with more beneficial mutations (that is, clonal interference).

And how many mutations have accumulated in the evolving genomes? Tenaillon *et al.* (2016) sequenced 264 clonal genomes from the LTEE (2 clones from 11 time points and 12 populations) and compared them with the ancestral genome to identify the mutations. In total, they found over 17 000 mutations, although most were in the six populations that evolved hypermutability. Clones sampled at generation 50 000 from populations that were not hypermutable averaged ~ 75 mutations in their genomes, in contrast to the billion-plus mutation events that occurred in each population. Those 75 or so mutations also pale in comparison with the differences between *E. coli* and *Salmonella* (Ochman *et al.*, 1999), or between two *E. coli* isolates from nature (Dixit *et al.*, 2015). However, it is not surprising because the time scale of the LTEE, while long for an experiment, is a drop in the bucket of evolutionary time.

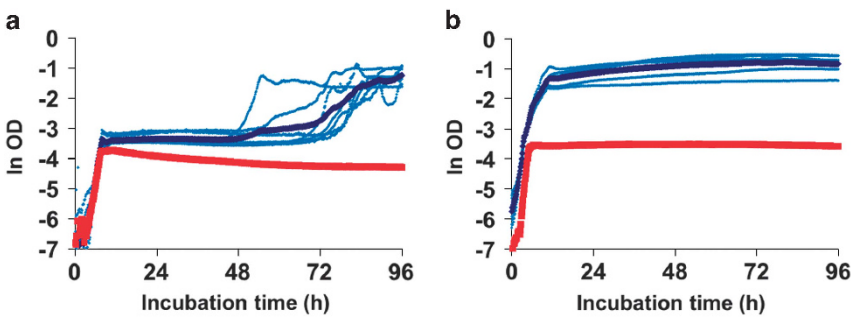

Figure 4 Growth curves of two Cit$^-$ clones, each with and without a plasmid carrying the evolved citrate-utilization module, on the medium used in the LTEE. OD, optical density, shown on a natural-logarithmic scale. (**a**) The ancestral strain of the LTEE. (**b**) A 32 000-generation clone closely related to the Cit$^+$ lineage that evolved in population Ara–3. The red and dark blue curves show average growth trajectories for the parent clone and its plasmid-bearing transformant, respectively; the light blue curves show replicates for the transformant. The ancestral strain's transformant experiences a long and variable delay between depletion of glucose and growth on citrate, whereas the evolved transformant with the same plasmid switches over quickly and efficiently. Modified from Blount *et al.* (2012).

Not all the mutations that accumulated in the LTEE genomes are beneficial—some might be neutral or even slightly deleterious mutations that 'hitchhiked' with beneficial mutations, and others new mutations that have hardly been tested by selection. However, two lines of evidence led Tenaillon *et al.* (2016) to conclude that the majority of the point mutations in the evolved clones from nonhypermutable lineages were, in fact, beneficial. First, the trajectory of accumulated mutations over time was better fit by a model with two parameters: a linear term describing the expected accumulation of neutral mutations; and a curvilinear term that describes the declining rate at which beneficial mutations arise and fix, as reflected in the declining rate of fitness improvement. Although the fraction of observed mutations that are beneficial falls as the second term becomes smaller over time, most mutations in the evolved genomes are beneficial based on this analysis. The second line of evidence compared the accumulation of nonsynonymous and synonymous mutations. After adjusting for the number of sites at risk for each type, Tenaillon *et al.* found an excess of nonsynonymous to synonymous mutations in the evolved genomes of ~17-fold over the first 500 generations of the LTEE and ~3.4-fold over 50 000 generations (Figure 5). The same approach revealed an excess of point mutations in intergenic regions of the evolved genomes.

Genetic targets of selection

The tandem duplication involving the *citT* gene was clearly important in one population. More generally, although, what genes have changed to improve fitness by ~70%, on average, after 50 000 generations? The approaches used to find the genetic targets of selection in the LTEE have changed over the years, with observable phenotypic alterations (Cooper *et al.*, 2001, 2003) and changes in DNA 'fingerprints' (Papadopoulos *et al.*, 1999; Schneider *et al.*, 2000; Woods *et al.*, 2006) providing some ways to track down and study mutations in years past. Today, as reported above, whole-genome sequencing allows one to find all of the mutations in a clone (Velicer *et al.*, 2006; Barrick *et al.*, 2009; Deatherage and Barrick, 2014), and whole-population metagenomic sequencing finds polymorphisms as well (Barrick and Lenski, 2009; Lang *et al.*, 2013). But not all of the mutations thus found are beneficial; some are mere hitchhikers. How can one separate the wheat from the chaff to infer the genetic targets of selection? One approach that has proven powerful in the LTEE (Cooper *et al.*, 2003; Woods *et al.*, 2006), as well as in other studies both experimental (Wichman *et al.*, 1999; Burke *et al.*, 2010) and comparative (Losos *et al.*, 1998; Lieberman *et al.*, 2011), is to look for parallel evolution, that is, similar changes that arose repeatedly in independently evolving populations.

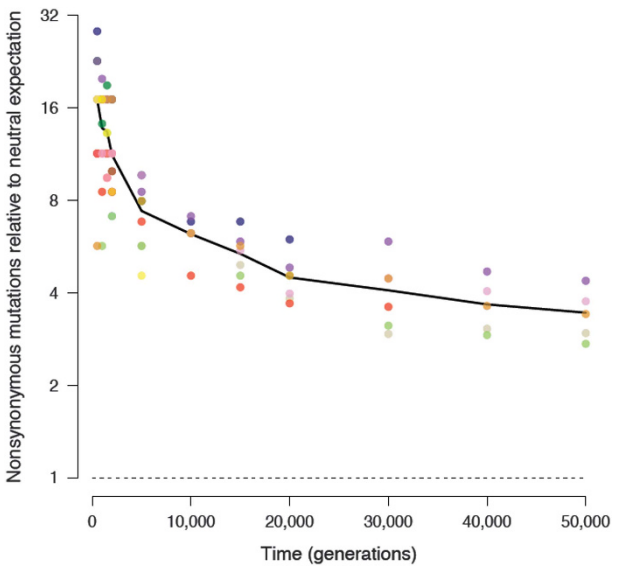

Figure 5 Accumulation of nonsynonymous mutations relative to the neutral expectation based on synonymous mutations, after adjusting for the numbers of genomic sites at risk for the two classes. Each point shows the average of two clones from one population that retained the ancestral mutation rate throughout or had not yet evolved hypermutability. The black line shows the mean trajectory. Modified from Tenaillon *et al.* (2016).

The hypermutability that evolved in some LTEE lineages complicates the analysis of parallel evolution by increasing the numbers of other mutations relative to the beneficial ones. Therefore, Tenaillon *et al.* (2016) focused on samples from populations that retained the ancestral mutation rate throughout and others before they became hypermutable. They found a strong signal of parallel evolution, with 57 genes that comprise only ~2% of the protein-coding portion of the genome having half of the nonsynonymous mutations in the evolved clones. Among the genes that showed the strongest signals of parallel changes are ones with core metabolic and regulatory functions including pyruvate kinase, peptidoglycan synthesis, DNA supercoiling and the stringent response. For several of these genes, isogenic strains that differ by a single mutation have been constructed and tested in competition, and the mutations were usually confirmed to be beneficial (Cooper *et al.*, 2003; Crozat *et al.*, 2005; Pelosi *et al.*, 2006; Barrick *et al.*, 2009). In most cases, however, the reason that a mutation is beneficial is not known. A plausible (but unproven) hypothesis is that many of these mutations fine-tune the regulation and expression of multiple functions to better match cellular requirements in the simple, predictable environment of the LTEE.

In addition to the parallel nonsynonymous substitutions, Tenaillon *et al.* (2016) also found numerous parallel deletions of genes and blocks of linked genes. Many of these genes were horizontally acquired before the start of the LTEE (that is, present in the ancestral strain), including remnants of prophages and toxin–antitoxin modules typically introduced by plasmids; some other deleted genes

encode host-specific functions including the production of extracellular capsules and fimbriae. The average genome size declined by ~1.4% over 50 000 generations. These deletions presumably eliminate unused but costly functions. However, many deletions were generated by recombination between repeated sequences, especially IS elements, which might indicate high-frequency events rather than (or in addition to) selection favouring the mutants (Cooper *et al.*, 2001).

Where do we go from here?

The future of the LTEE

My hope is that the LTEE will continue for many generations of scientists (Fox and Lenski, 2015). The LTEE is simple and fairly inexpensive to run. More importantly, there are good reasons to keep it going. First, by its very nature evolution takes time, and some changes—the origin of new functions and even species, for example—typically require a lot of time. So the longer the LTEE runs, the more we can learn. In a related vein, evolving systems are clever, so there are potential surprises—changes that might seem unlikely at the outset or that would not even have been imagined. The evolution of citrate use is probably the biggest change observed in the LTEE to date, but one can imagine bigger changes. Could a cross-feeding interaction, where one ecotype consumes a by-product released by another, evolve into a predator–prey interaction, where one party kills the other to get more of the product? Or might sex evolve, either by reactivating a non-functional prophage or acquiring the ability to take up DNA from lysed cells? Second, new technologies may enable one to look at evolutionary changes in novel ways. Just as it became possible to sequence entire genomes, perhaps one day it will be possible to image, analyze, and understand the entire metabolism of a cell through time. Third, many talented people have worked on the LTEE, and they bring new ideas and approaches. I am confident that future generations of scientists will be equally talented and innovative.

Other evolution experiments of interest to microbial ecologists

Of course, the LTEE is just one experiment, and there have been many other fascinating evolution experiments with microbes. I cannot do justice to all the questions addressed and the diversity of systems used in these experiments. However, let me briefly highlight several dozen articles that I think many microbial ecologists will find interesting. They cover so many different topics that I will simply list them in chronological order.

- Chao *et al.* (1977) showed that coevolution of bacteriophage T7 and its *E. coli* prey led to rapid diversification and an increasingly complex community structure.
- Paquin and Adams (1983) saw that non-transitive competitive interactions in evolving populations of *Saccharomyces cerevisiae* caused declines in fitness relative to the initial strain, although genotypes that rose to dominance were more fit than their immediate progenitors.
- Bull *et al.* (1991) showed that a filamentous phage evolved reduced virulence when its transmission was strictly vertical, but the vertically adapted lines were outcompeted during horizontal transmission.
- Zambrano *et al.* (1993) found that evolution during prolonged starvation favored *E. coli* mutants that can grow when other cells are in stationary phase or even dying.
- Rosenzweig *et al.* (1994) observed the evolution of stable polymorphisms mediated by cross-feeding interactions in chemostat cultures of *E. coli*.
- Schrag *et al.* (1997) showed that antibiotic-resistant *E. coli*, when propagated without antibiotic, may evolve compensatory mutations that eliminate the cost of resistance and even preclude reversion to sensitivity.
- Rainey and Travisano (1998) demonstrated that spatially structured environments and the resulting physicochemical gradients promoted diversification of *Pseudomonas fluorescens* into multiple ecotypes that exploited distinct niches.
- Burch and Chao (1999) found that populations of an RNA phage followed different evolutionary paths depending on their population size during periodic bottlenecks.
- Wichman *et al.* (2000) evolved phages ϕX174 and S13 on both *E. coli* and *Salmonella typhimurium* hosts and saw that many of the resulting substitutions affected the same codons that distinguish these closely related phages, indicating convergence between natural and laboratory-evolved lineages.
- Dionisio *et al.* (2005) evolved a multidrug-resistant plasmid that enhanced the fitness of its *E. coli* hosts even in the absence of antibiotics.
- Dennehy *et al.* (2006) examined how source-sink dynamics affected the potential for a virus to adapt to a new host type.
- Fiegna *et al.* (2006) started with a mutant of *Myxococcus xanthus* that was an obligate cheater —able to form fruiting bodies and spores only by exploiting other strains—and evolved a strain that not only makes fruiting bodies and spores but also resists cheating.
- Gerstein *et al.* (2006) evolved haploid, diploid and tetraploid strains of *S. cerevisiae* and found they converged on diploidy.
- Kerr *et al.* (2006) showed that restricted migration of phage T4 promoted the evolution of more prudent use of their bacterial prey, whereas unrestricted migration favored more rapacious phage.

- Moore and Woods (2006) compared rates of adaptation to a laboratory environment among several strains of *E. coli* isolated from natural hosts.
- Cooper (2007) showed that horizontal gene transfer accelerated adaptive evolution by reducing clonal interference between beneficial mutations in *E. coli* populations.
- Meyer and Kassen (2007) found that predation by the protist *Tetrahymena thermophila* eventually increased the diversity of its bacterial prey, but it also delayed diversification by lowering the prey population size and hence the supply of mutations.
- Tagkopoulos *et al.* (2008) evolved *E. coli* in a regime where an increase in temperature preceded a shift from anaerobic to aerobic growth, rather than the opposite shift that occurs when cells colonize the gut, thereby reversing the coupling in gene expression.
- Hillesland and Stahl (2010) saw the evolution of increased stability and productivity in a constructed mutualism between the sulfate-reducing bacterium *Desulfovibrio vulgaris* and the methanogenic archaeon *Methanococcus maripaludis*.
- Paterson *et al.* (2010) showed that genome evolution in phage ϕ2 was faster when its *P. fluorescens* prey were allowed to coevolve than when the prey population was kept constant by restarting it from frozen samples.
- Sota *et al.* (2010) propagated antibiotic resistance plasmids in several host species and found that the plasmids evolved to be more stably inherited.
- Kuzdzal-Fick *et al.* (2011) cultured *Dictyostelium discoideum* under different regimes and showed that cheaters that exploit other strains evolved under conditions of low, but not high, genetic relatedness.
- Poltak and Cooper (2011) evolved *Burkholderia cenocepacia* biofilms and observed the emergence of heterogeneous mixtures that were more productive than any individual member.
- Bell (2012) propagated hundreds of populations of the green alga *Chlamydomonas* in the dark and found most went extinct, but a history of sexual reproduction increased the proportion of surviving lineages.
- Ensminger *et al.* (2012) maintained *Legionella pneumophila* in mouse macrophages and showed that some mutations beneficial in that environment were detrimental in their usual protozoan hosts.
- Meyer *et al.* (2012) found that coevolution of phage λ and *E. coli* often favored phage genotypes that could infect cells through a novel receptor, one not previously detected in traditional genetic work on this interaction.
- Ratcliff *et al.* (2012) used a simple size-based selection scheme to evolve *S. cerevisiae* that undergo a multicellular life cycle.
- Soto *et al.* (2012) studied adaptation of the bioluminescent *Vibrio fischeri* to alternative species of squid hosts.
- Tenaillon *et al.* (2012) evolved >100 replicate *E. coli* lines at high temperature and, based on whole-genome sequencing, found two distinct adaptive pathways.
- Toprak *et al.* (2012) evolved *E. coli* populations that grow at increasing concentrations of antibiotics using a 'morbidostat' that maintains a constant level of inhibition.
- Bachmann *et al.* (2013) used a water-in-oil emulsion to produce a population structure that favored higher yield, as opposed to faster growth, in *Lactococcus lactis*.
- Lang *et al.* (2013) performed metagenomic sequencing on 40 evolving populations of *S. cerevisiae* to characterize the forces responsible for the rise and fall of polymorphisms.
- Lindsey *et al.* (2013) showed that rapidly rising antibiotic concentrations drove *E. coli* populations extinct by precluding certain evolutionary paths that would be accessible if the changes were slower.
- Traverse *et al.* (2013) evolved *B. cenocepacia* under a regime that favored both biofilm formation and dispersal, finding mutations similar to those in isolates of pathogens that colonize the lungs of individuals with cystic fibrosis.
- Van Ditmarsch *et al.* (2013) saw the evolution of multi-flagellated, hyper-swarming *P. aeruginosa*, with a concomitant reduction in their ability to form biofilms.
- Hutchins *et al.* (2015) showed that the marine cyanobacterium *Trichodesmium*, which increases its nitrogen fixation as a plastic response to high CO_2, evolved a constitutively elevated rate after several years of exposure to that condition.
- Levy *et al.* (2015) used random barcodes to track lineages in *S. cerevisiae* populations, revealing thousands of beneficial mutations that transiently increased in abundance but were eventually outcompeted by other more beneficial mutations.
- Scheinin *et al.* (2015) performed an evolution experiment using mesocosms in a marine environment with both ambient and elevated CO_2 levels, and they saw adaptation of the diatom *Skeletonema marinoi* to the elevated concentration.
- Baym *et al.* (2016) constructed devices—in essence, giant Petri dishes—on which they could directly visualize the evolution of motile *E. coli* able to migrate into and grow at increasing antibiotic concentrations.
- Meyer *et al.* (2016) studied the divergence and incipient speciation of a phage λ strain that could infect *E. coli* through either of two receptors into specialists able to use only one or the other.
- Schlüter *et al.* (2016) demonstrated a complicated interplay between the physiological and evolutionary responses of the calcifying phytoplankton *Emiliania huxleyi* to ocean acidification.

- Kacar *et al.* (2017) mixed paleobiology, synthetic biology and experimental evolution by making an ancient version of a core gene, moving it into the chromosome of a modern *E. coli* strain, and observing how the bacteria subsequently evolved.

Also, several review articles and books discuss these and other evolution experiments that have been performed using microorganisms as well as animals and plants (Bell, 1997; Elena and Lenski, 2003; Buckling *et al.*, 2009; Garland and Rose, 2009; Kawecki *et al.*, 2012; Barrick and Lenski, 2013; Brockhurst and Koskella, 2013; Kassen, 2014; Hoang *et al.*, 2016).

Opportunities to integrate experimental evolution and microbial ecology

Even with this extraordinary range of topics, there are undoubtedly more opportunities to use experimental evolution to address issues of interest to microbial ecologists, evolutionary biologists and geneticists. Also, most experiments lasted only a few hundred generations. I think much more could be learned by substantially extending such experiments. Evolution is an open-ended process that often involves complex ecological feedbacks and genetic interactions. Given time, these feedbacks and interactions may yield new discoveries and interesting surprises, as has happened throughout the LTEE. I do not mean to minimize the difficulty or costs, but I suggest that LTEEs might sometimes run in the background as teams pursue other short-term projects. With the ability to freeze samples and the declining costs of many high-throughput analyses, such long-term experiments could prove cost-effective in terms of their scientific value.

In closing, let me propose a strategy that might build an interesting and productive new bridge between experimental evolution and microbial ecology. To date, most evolution experiments have examined either single species evolving in isolation, like the LTEE, or pairs of interacting species, such as bacteria and phage. In principle, we would like to watch an entire microbial community of thousands of species evolve over time, with the potential to replicate communities, freeze all of the constituent members, manipulate genomes and environments in order to distinguish adaptation from random drift, and more. But it is hard to know where to begin in designing, much less analyzing, such a daunting experiment. The following strategy, by contrast, extends experimental evolution to whole communities, but in a way that limits the conceptual and practical difficulties. We might call this generic approach an 'embedded species evolving in a natural community experiment'—or ESENCE for short.

An ESENCE would begin by identifying one member of a natural community that is the primary focus of the experiment. That species would be chosen, at least in part, based on its experimental tractability including the availability of genetic markers that allow it to be tracked and enumerated (without handicapping its performance in the community) as well as the potential to manipulate its genome to evaluate the effects of mutations that arise during the ESENCE. Rather than trying to understand the entire community and its evolution from the outset, one could (1) isolate a representative of the focal species from the community, (2) mark the isolate genetically so that it can be monitored, (3) introduce the marked isolate and its unmarked progenitor into replicate microcosms subject to the same or different treatments of interest, (4) monitor the relative abundance of the marked and unmarked variants of the focal species, (5) use a shift in their relative abundance as an indication that the variant that is increasing in relative abundance has acquired a beneficial mutation (De Visser and Rozen, 2006; Hegreness *et al.*, 2006; Kao and Sherlock, 2008; Woods *et al.*, 2011), (6) sample both variants and sequence their genomes, ideally finding they are identical except for one or a few mutations—or gene transfer events (Shapiro *et al.*, 2012; Bendall *et al.*, 2016)—of interest. If multiple changes occurred, one could (7) make isogenic strains to separate the mutations, using parallelism across replicates (Wichman *et al.*, 1999; Lieberman *et al.*, 2011; Tenaillon *et al.*, 2016) or other information to identify changes of particular interest. One could then (8) compete the constructed strains to test whether a mutation of interest is beneficial in the context of the community and treatment imposed and, (9) at the same time, monitor the effects of the changing abundances of the manipulated competitors on other community members, thereby beginning to probe and identify eco-evolutionary feedbacks between the focal species and the community (Widder *et al.*, 2016). If two or more treatments (for example, different environments) are used, then one could also (10) test an evolved mutation in the other treatments as well as the one in which it arose to evaluate whether its benefit, if any, was specific to the relevant treatment.

Performing an ESENCE will certainly be difficult, but the experiment by Scheinin *et al.* (2015) on adaptation of an ocean-dwelling diatom to increased CO_2 level shows the potential for this approach. One difference between that study and the ESENCE strategy is that evolution depended on standing genetic variation present at the start of the experiment, rather than on de novo variants. That difference was unavoidable given the study system, but it does complicate genetic analysis. Gómez and Buckling (2011, 2013) performed an experiment in which they compared the coevolution and diversification of *P. fluorescens* and phage SBW25ϕ2 between soil microcosms with and without the resident microbial community, anticipating many aspects of the ESENCE strategy outlined above. These experiments—along with beautiful studies on evolution in action in natural systems with

limited diversity, including acid-mine drainage communities (Denef *et al*., 2010) and the lungs of people with cystic fibrosis (Lieberman *et al*., 2011)—should inspire and stimulate future directions in the field of microbial experimental evolution.

Conflict of Interest

The author declares no conflict of interest.

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