ISSN 0006-2979, Biochemistry (Moscow), 2025, Vol. 90, No. 11, pp. 1454-1467 © Pleiades Publishing, Ltd., 2025.
Russian Text © The Author(s), 2025, published in Biokhimiya, 2025, Vol. 90, No. 11, pp. 1544-1560.

REVIEW

Laboratory Evolution: Molecular–Genetic Basis and Phenotypic Plasticity

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Received May 21, 2025
Revised August 19, 2025
Accepted September 9, 2025

Abstract—Adaptive laboratory evolution (ALE) is aimed at elucidating the molecular basis of adaptation and is widely employed as a tool for gaining deeper insight into genetic and/or metabolic pathways underlying evolutionary processes. One of the primary goals of experimental evolution is to predict mutations representing the key driving forces of adaptation. The use of whole-genome resequencing enables easy identification of mutations that arise during ALE, and consequently, biochemical alterations that occur in the experimental lineages. ALE has also proven highly relevant in practical applications, as it provides an innovative approach to the construction of evolved microbial strains with desirable performance, such as rapid growth, stress resistance, efficient utilization of diverse substrates, and production of compounds with a high added value (amino acids, ethanol, aromatic compounds, lipids, etc.). In this review, we analyzed the results of studies focused on the demonstration and explanation of relationships between mutations and resulting phenotypic and biochemical changes, as well as discussed a potential of microorganisms as model systems for ALE experiments and testing of various evolutionary hypotheses. We also described achievements reached by using ALE strategies, as well as the still unresolved issues and methodological limitations of this approach.

DOI: 10.1134/S0006297925601583

Keywords: adaptation, laboratory evolution, metabolic engineering, microorganisms, causal mutations

INTRODUCTION

Understanding the mechanisms by which organisms respond to global crises, such as climate and ecosystem changes, spread of invasive species, emergence of multidrug-resistant pathogens, and a growing demand for food, has become progressively important for predicting evolution and interpreting its dynamics. In nature, evolution can be driven by various factors, including environmental shifts or isolation of small populations. However, assessing the predictability of evolution solely from the observational studies of natural populations is rather challenging, as the evolutionary history cannot be experimentally replicated. To overcome these limitations, scientists have turned to laboratory evolution experiments, primarily using microbial populations [1], since they provide simple model systems in which multiple replicate populations can be propagated for hundreds or thousands of generations, allowing precise quantification of mutation frequencies over time under controlled and manipulable environmental conditions. This approach can contribute much to the insight into the mechanisms of adaptation and genome evolution. Hence, experimental laboratory evolution studies aim to observe living organisms in controlled scenarios that promote evolutionary changes and enable investigation of the molecular basis of adaptation. Although controlled laboratory environment does not always fully represent natural ecological conditions, adaptive laboratory evolution (ALE) studies have proven highly successful in substantiating evolutionary theories based on actual molecular and mechanistic evolutionary models [2, 3]. By combining phenotypic characterization and genome sequencing of the evolved experimental lineages, it has become possible to observe evolution

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in real time and to address the question of whether certain phenotypic and genotypic results can be predicted. In a stable environment, the rate of fitness improvement slows down as populations become more adapted [4]. However, improvement in fitness never ceases completely, even it becomes markedly slower. If we assume that the representatives of different generations enter into direct competition, younger generations might exhibit higher adaptive fitness or possess greater resources, which gives them a competitive advantage. However, such hypothesis requires to take into account multiple factors, including environmental conditions, available resources, and specific abilities characteristic of each generation [2, 5]. Adaptive traits resulting from mutations acquired in the process of laboratory evolution may manifest themselves in various ways, e.g., as increased tolerance to specific stressors, ability to utilize non-natural substrates, or more efficient metabolism of conventional substrates [6-11]. At the same time, the rate of molecular evolution (i.e., mutation fixation) tends to remain relatively constant [12-14].

Experimental studies of microbial evolution have a long-standing history. The concept of a continuous evolutionary experiment achievable by using standard laboratory methods can be traced back to the 19th century, following the publication of Charles Darwin's works. A contemporary of Darwin, the Reverend Dr. William Dallinger, who was also a microbiologist, conducted experiments on the unicellular flagellated eukaryotes Cytomonads [15]. Over seven years, he had succeeded in gradually adapting three Cytomonads species to a temperature increase from 16°C (60°F) to 78°C (155°F). The initial cultures felt comfortable at 65°F but perished at 140°F. This work provided the first demonstration that microbial populations can undergo significant adaptive changes under laboratory conditions within a relatively short period of time. The number of studies on ALE has significantly increased over the past decade [16]. At the current stage of biology development, a keen interest in laboratory evolution has become a result of development of relatively inexpensive next-generation sequencing technologies, which enable detailed comparison between the experimental and ancestral lineages. Whole-genome resequencing of experimental and ancestral lineages has revealed that phenotypic adaptation is accompanied by a continuous process of mutation accumulation, extensive genetic parallelism, and a pronounced historical contingency [2, 3, 17]. In many cases, the effects of *de novo* mutations inferred from bioinformatic analysis, can be validated using molecular methods.

Mutations range from single-nucleotide polymorphisms and small indels (insertions or deletions) in genes encoding specific enzymes or transporters that facilitate utilization of new substrates or more efficient uptake and channeling of substrates into the central metabolism) to regulatory mutations that restore the metabolic network balance by disabling nonessential growth functions and releasing resources for processes directly related to growth (e.g., substrate uptake) [9, 10, 18-21].

This goal of our review was to demonstrate the relationship between mutations fixed in the course of ALE experiments and corresponding phenotypic and biochemical changes, as well as the potential of microorganisms as model systems in laboratory evolution experiments aimed at investigating adaptation processes, estimating evolutionary parameters, and testing various evolutionary hypotheses. It is important to mention the already obtained results not only demonstrate the capacity and versatility of the ALE approach, but also demonstrate its current limitations, as well as open questions that required further investigation.

ADVANTAGES OF BACTERIA AND FUNGI AS MODEL ORGANISMS IN ALE

Microorganisms are well-suited for the ALE experiments for several reasons: their short generation times allow to study the evolutionary dynamics across many generations within a relatively short time period; they can easily sustain large population sizes; many bacterial species can be stored in a dormant state for further investigation, etc. This enables researchers to "go back in time" and directly compare evolved strains with their ancestors [22]. Most experimental evolution studies use unicellular organisms, such as *Escherichia coli*, due to their rapid growth, short reproduction time, small genome size, ability to adapt to environments with certain nutrients and/or stressors, and their status as model organisms, which provides an appropriate body of knowledge for interpreting experimental results. Importantly, most microbial cells have simple nutrient requirements and are easy to cultivate under laboratory conditions. A high cell division rate and a relatively small genome size of unicellular organisms also make it feasible to sequence multiple clones or microbial population samples from different time points at a low cost, thus providing high resolution for detecting genetic changes associated with observed evolutionary processes. In a typical microbial evolution experiment, cells are inoculated into a medium and allowed to grow until the culture reaches a high population density. Next, they are transferred to a fresh medium or diluted to enable further growth and division. This cycle can continue indefinitely, and over successive generations, natural selection results in the adaptation to the laboratory

environment. Establishment of conditions exceeding those providing the optimum performance of a strain, will stimulate adaptation. The range of environmental parameters that can be used for driving the selection are very broad; as long as a chosen condition imposes selective pressure that determines differential survival within a population, adaptation will occur.

E. coli, which has a generation time of only about 20 min, is easy to cultivate with a high yield, and its gene expression in this bacterium can be efficiently controlled, making *E. coli* an undoubtedly convenient model organism for experimental evolution [9, 23-25]. However, bacteria are highly sensitive to low pH and presence of sulfur dioxide in the medium, as well as lack of the ability to perform post-translational modifications. Hence, the range of questions that can be explored in these microorganisms is limited by their relative simplicity. Eukaryotes, such as fungi or protists, with their more complex gene regulation, morphology, and physiology, provide opportunities to address a much broader spectrum of evolutionary questions [26].

Fungi occupy a unique niche among eukaryotic systems. Because of the short generation times, compact genomes, and sexual life cycles, they represent a valuable and still largely underutilized resource for advancing experimental evolution research. Fungi are convenient subjects for ALE experiments for several reasons. They are generally characterized by simple and rapid life cycles and can produce hundreds to thousands of generations in a relatively short time period, and their large effective population sizes are comparable to those of bacteria and reflect the sizes of natural population. Fungi exhibit high tolerance to acidic environments; they have a larger cell size, can perform post-transcriptional protein modifications (such as glycosylation), and possess effective stress adaptation mechanisms. Many fungal species grow on agar media and are cryotolerant. Fungi can reproduce both as haploid and diploid organisms, sexually and asexually. They are easy to cross, and the progeny can be easily separated from the parental cells. Most original population can be limited to a single cell to provide genetic uniformity at the beginning of the experiment. Therefore, fungi have simple genomes and fast cell cycles typical of other microorganisms, but with the eukaryotic complexity unattainable in bacterial systems [27]. In this regard, fungi are perfect model organisms. Furthermore, they have a simple morphology, are easy to experiment on, inhabit distinct and well-characterized ecological niches, and show remarkable diversity, ranging from saprotrophs to pathogens, mutualists, and even predators [28].

Sexual reproduction is widespread in nature, and almost all organisms engage in some form of genetic exchange. However, most experimental evolution studies have been focused on asexual systems, leaving the role of recombination in the adaptation largely unexplored. Fungi can serve as the optimal model systems for addressing this question. Direct comparison of the adaptation rates and mutations evolved in sexual and asexual populations of *Saccharomyces cerevisiae* has shown that sex not only promoted adaptation but also enabled natural selection to more efficiently separate beneficial mutations from deleterious ones. Sexual reproduction combines advantageous mutations from different lineages in the same genome, reduces clonal interference, and restores beneficial mutations from deleterious backgrounds [27, 29]. When comparing adaptation rates to a new environment over 3000 mitotic generations, it was demonstrated that in *Aspergillus nidulans*, somatic growth involves mitotic recombination at an extremely high rate, which reinforces adaptation to the new environmental conditions [30]. Another potential advantage of recombination is resolution of clonal interference. In populations lacking recombination, different beneficial mutations cannot be combined within a single genetic background, whereas in sexual populations, all measured advantageous mutations had been fixed [29, 31].

Fungal systems used in experimental evolution studies include unicellular yeasts of the *Saccharomyces*, *Schizosaccharomyces*, and *Candida* genera, filamentous basidiomycetes (*Schizophyllum* and *Ustilago*), and ascomycetes (*Neurospora* and *Aspergillus*). Recently, new species, such as the halotolerant *Hortaea werneckii* black yeast [32] and short-lived filamentous ascomycete *Podospora anserina* have been added to the models used in ALE studies [26, 33]. It should be noted that filamentous fungi remain underrepresented in this field, as the development of genetic engineering tools for these organisms is hindered by the high complexity of their genomes and associated metabolic and cellular processes. Challenges include slow rates of mycelium growth, low production yields, suboptimal accumulation of alternative products, and difficulties in purification. Nevertheless, several interesting experiments have been carried out in *Penicillium commune* and *Penicillium* sp. [34], as well as in *Mortierella elongata* co-cultured with the alga *Nannochloropsis oceanica* [35], although specific genetic mechanisms controlling the observed evolution of traits in *Penicillium* and *Mortierella* have not yet been identified.

SHORT-TERM ALE EXPERIMENTS

The main goal of short-term ALE experiments is to predict which mutations are “significant” drivers of adaptation. Using this methodology, it was shown

that gene loss can enhance an organism's ability to evolve and adapt by providing alternative evolutionary pathways. Although gene deletions often lead to an immediate reduction in fitness, many mutants rapidly acquire suppressor mutations that restore fitness, and some even surpass the fitness levels of similarly adapted wild-type cells [36]. The observed high frequency in the loss-of-function mutations suggests that these mutations play a major role in bacterial adaptation to new environments and may provide substantial fitness advantages under various stressful conditions. In most short-term ALE experiments (74%), the frequency of nonsense mutations exceeded that of missense mutations, and this increased rate of gene-inactivating nonsense mutations was due to their selective advantage (i.e., beneficial effect). The authors concluded that the loss-of-function mutations that escape purifying selection are major drivers of adaptation during short-term evolution [37].

ALE has been extensively used to explore the genetic and biochemical basis of bacterial and fungal adaptation. Whole-genome resequencing enables easy identification of mutations that arise during ALE and, consequently, biochemical changes occurring in experimental lineages. For instance, short-term ALE was successfully applied to improve carotenoid production in an engineered *S. cerevisiae* strain using periodic hydrogen peroxide shock cycles, resulting in a threefold increase in the carotenoid yield. Subsequent transcriptomic analysis aimed at elucidating molecular mechanisms underlying this improvement revealed activation of genes involved in lipid metabolism and mevalonate biosynthesis pathway in hyperproducing strains [38]. The tolerance of *Rhodococcus opacus* to phenol, a model product of lignin degradation, was achieved through serial cultivation in the media containing phenol as the sole carbon source, followed by screening for fast-growing mutants. After 40 passages, some strains exhibited enhanced phenol tolerance. Whole-genome sequencing of the experimental strains to identify genomic alterations that had appeared during adaptive evolution, combined with comparative transcriptomics to detect transcriptional changes, proved to be effective for revealing the tolerance mechanisms and identifying promising candidate genes to facilitate future metabolic engineering of *Rhodococcus*. The adapted strains demonstrated higher phenol consumption rates and approximately twofold increase in the lipid production from phenol compared to the wild-type strain. Due to the consistent identification of single-nucleotide polymorphisms in two transporter/permease genes, it was suggested that the adapted strains had altered transport of phenol or related compounds. Genes involved in the phenol conversion to catechol were among the most upregulated when the strains were grown on phenol vs. glucose, indicating that phenol-to-catechol conversion may represent a rate-limiting factor for the growth on phenol [39]. It was discovered that through ~350 generations of laboratory evolution, the early evolutionary response (tolerance) of *E. coli* cells to the antibiotic trimethoprim included derepression of signaling through the Mg^{2+}-sensitive two-component PhoPQ system achieved via inactivation of the negative feedback regulator MgrB. It was suggested that mutations in the *mgrB* gene precede and promote the development of antibiotic resistance in bacteria [40].

Several studies have investigated the early stages of evolution of multicellularity from unicellular ancestors using experimentally evolved yeasts exhibiting a "snowflake" phenotype (formation of clusters by yeast cells). These yeasts have acquired the ability to cluster through inactivation of the *trans*-acting transcription factor ACE2 [41], which regulates expression of enzymes required for the separation of mother and daughter cells after mitosis [42]. As a result, daughter cells remain attached to the mother cells, producing a branched, snowflake-like phenotype. ALE was used to directly study the evolution of early multicellularity, including transition from the cell-level selection to the cluster-level selection, as well as the development of cellular differentiation. Because cell clusters settle faster in liquid media than individual cells, primitive multicellular forms of *S. cerevisiae* were selected using gravity, which allowed straightforward selection of genotypes that formed the clusters. After 60 transfers of settled cells into fresh medium, all selected populations exhibited approximately spherical, snowflake-like phenotypes consisting of multiple adherent cells. Since "snowflake" yeasts were formed due to the adhesion between the mother and daughter cells after cell division, they exhibited a high genetic uniformity within the clusters, thus reducing potential conflicts of interest among constituent cells. The snowflake phenotype was stable: after 35 transfers without gravitational selection, populations derived from day 30 of the first evolutionary experiment showed no invasion by any unicellular strains. Cell differentiation is a characteristic feature of multicellular organisms, and some cells were required to compromise their reproductive potential for the efficient reproduction of the whole cluster. Presumably, the evolution of cellular differentiation has occurred due to apoptosis, which snowflake yeasts underwent to generate more numerous, smaller (and thus faster-growing) propagules compared with the parental cluster. In this experimental system, most cells remained viable and capable of reproduction, while some underwent apoptosis, which is functionally analogous to the germ-soma differentiation, when cells specialize in the reproductive and nonreproductive roles [43, 44]. Apoptotic cells functioned as the breaking points within multicellular

clusters, allowing snowflake yeasts to produce more propagules from a given number of cells. Seven-day-old yeasts formed relatively small clusters with a low level of apoptosis, whereas 60-day-old yeasts formed larger clusters with elevated apoptosis [45]. This higher rate of apoptosis observed in the late-stage yeast isolates could not be explained by diffusion gradients caused by multicellularity but rather appeared to evolve in parallel with the increase in the cluster size, possibly because larger clusters tended to contain a higher proportion of aged cells, which underwent apoptosis at a higher rate [46].

Directed evolution of approximately 800 generations of snowflake yeast combined with mathematical modeling provided insights into the influence of transition to aerobic metabolism on multicellularity [47]. Although oxygen provides substantial metabolic advantages, the shift from anaerobic to microaerobic conditions constrains the evolution of larger body sizes because at low concentrations, oxygen cannot diffuse deeply into the tissues. As the availability of oxygen in the environment increases, larger organisms gain selective advantage, as deeper oxygen penetration alleviates a compromise between the growth rate and body size [47].

Pineau et al. [48] observed the development of two distinct phenotypes – small and large snowflake yeasts – from a single clonal ancestor. Their coexistence resulted from the ecological specialization: small snowflakes evolved as the growth-rate specialists, whereas genotypes forming 16-48 times larger clusters evolved as survival specialists. The compromise between the size and resource competition maintained their long-term coexistence. This oxygen availability-based compromise was essential for maintaining the diversity, as the coexistence was disrupted when extra oxygen was supplied and has never developed in the mixotrophic and anaerobic populations during ALE. The authors suggested that a simple compromise between the growth and survival, established by different extent of oxygen diffusion through the bodies of varying sizes, may promote and maintain ecological diversity in emerging multicellular lineages [48].

Most microbial evolution experiments, as well as the studies in multicellular eukaryotes, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, include no more than 1000 generations of adaptation to a new environment [1]. This makes them suitable for studying the early dynamics of adaptation, when a population encounters a novel environment for the first time and rapidly accumulates beneficial mutations in response to the new challenge. However, many researchers have questioned how far such results can be extrapolated. Will evolutionary dynamics remain the same over longer periods of time? Can it change qualitatively after thousands of generations of adaptation to laboratory conditions? These issues motivated the development of long-term ALE experiments.

LONG-TERM EVOLUTION EXPERIMENTS

The experiment initiated by Richard Lenski has become a universally recognized standard in the field. The critical factor that determined the success of this work was the choice of one of the simplest, most convenient, and best-studied laboratory organisms – *E. coli* bacterium. The experiment began in February 1988 and has now been running for 37 years, allowing the author to draw certain conclusions and answer a number of frequently asked questions [49]. This work has become known as the long-term evolution experiment (LTEE). The simplicity of *E. coli* cultivation, the use of 12 parallel experimental lineages grown under identical conditions, and the employment of periodic batch culture ensuring indefinite continuation of the experiment, together with a rigorous protocol involving daily transfers of 1% of each population, cryopreservation of samples every 75 days, application of modern genomic analysis techniques, and ability to branch off new experiments at any time – all these factors have contributed to the LTEE success and allowed numerous co-authors to obtain experimental evidence supporting key evolutionary hypotheses [50-52]. During the first 26,000 generations, the results followed the classical theory – random mutations occurred, with beneficial ones accumulating and deleterious ones being purged. After 26,000 generations, however, the mutation rate rose sharply due to alterations in the mutator gene responsible for the DNA error correction (any gene involved in DNA replication or repair may serve as such if slightly impaired). Overall, this effect is deleterious, since most mutations are harmful, but it also increases the probability of rare beneficial mutations. This becomes especially important once all high-probability beneficial mutations had already occurred. The mutator gene and the beneficial mutation are inherited together due to their physical linkage on the same chromosome, a process known as “hitchhiking.” Thus, selection for the beneficial mutation simultaneously maintains the mutator gene (via linked inheritance). Some obtained results were entirely unexpected [13].

Despite a considerable time investment, the need for continuous and laborious monitoring of cells, strict aseptic conditions to prevent contamination, and a requirement for direct relationship between the desired function and benefits to the organism, Lenski’s work demonstrated that extending the duration of evolutionary experiments can indeed lead

to additional valuable outcomes. Thus, only a few decades ago, it had been believed that the optimal adaptation to specified environmental conditions, such as those of LTEE, would be achieved by a bacterial population within only a few thousand generations [31]. However, reality proved otherwise: even after 60,000 generations (according to the Lenski's experiment), *E. coli* populations continued to adapt, and this was observed in all parallel lineages [13]. In LTEE, the rate of fitness increase in *E. coli* follows a power law, suggesting that there is no optimal adaptation that could be achieved by an evolving population [31].

Another example is the ability to utilize citrate from the growth medium under aerobic conditions, which unexpectedly emerged in one experimental *E. coli* lineage. This ability had been formed gradually during many preceding generations and manifested itself after ~31,000 generations [53]. The evolution of the cit^+ phenotype is particularly significant because the inability to metabolize citrate aerobically is a defining characteristic of *E. coli* as a species. Recent studies have shown that even after 2500 generations of adaptive evolution, the cit^+ trait causes the death of a large fraction of cells cultivated aerobically on citrate as the only carbon source, thus highlighting an inherent incompatibility between the aerobic citrate metabolism and stable *E. coli* physiology [54]. The effect of the key mutation enabling citrate transport into a cell under aerobic conditions, i.e., cit^+ phenotype, was found to depend on other "potentiating" mutations. These mutations did not affect citrate utilization directly, but instead provided genetic or physiological basis enabling manifestation of the key mutation, a process known as potentiation. Such additional mutations may be neutral or even beneficial for other functions (pre-adaptation), with a side effect in a form of ability for citrate utilization. This demonstrates that complex phenotypic evolutionary changes often require interactions among multiple mutations and "preparatory" alterations in the genetic background. The cit^+ phenotype (actualization stage) could be obtained only through mutation accumulation in a course of many bacterial generations. In other words, this trait is unlikely to develop during a short-term experiment [31]. Further evolutionary process provided increase in the citrate utilization efficiency due to novel optimization mutations (refinement stage). The authors suggested that the development of highly novel traits trough complex evolutionary trajectories may be akin to the processes occurring during speciation. Notably, none of the remaining 11 lines had evolved a similar ability even after 75,000 generations [2].

The studies on citrate utilization have elucidated the nature of observed limitations: the appearance of the key mutation depended on prior, random evolutionary changes. This explains why the new function emerged only after 31,000 generations and in only one of 12 replicate populations [2, 53].

Following Lenski, other researchers have adopted the term LTEE for their own long-term projects. Although none of them has been at the timescale of the Lenski's experiment, several investigations have exceeded most previous studies in duration, encompassing over a thousand generations. For example, Behringer et al. [55] had maintained 100 *E. coli* populations over 10,000 generations in tubes under conditions allowing both spatial and nutritional specialization. They observed repeated evolution of biofilm-forming phenotypes and stable coexistence of subpopulations and analyzed possible reasons for the stable coexistence of multiple dominant haplotypes over thousands of generations. They also identified a substantial number of parallel mutations among replicate populations. Fisher et al. [56] had maintained laboratory populations of budding yeast *S. cerevisiae* for 4000 generations and found that, similarly to *E. coli*, these populations acquired fitness along predictable trajectories characterized by the declining adaptivity. One of the most intriguing results of laboratory evolution experiments initiated with haploid yeast populations was the emergence of diploid lineages via whole-genome duplication. Tracking recurrent genome duplication across 46 haploid yeast populations evolving over 4000 generations revealed that autodiploids had been fixed already by generation 1000 in all 46 populations. Whole-genome duplication led to a decline in the adaptation rate, indicating a compromise between immediate fitness improvement and long-term adaptivity. The presence of ploidy-enriched targets of selection and structural variants showed that autodiploids can access adaptive pathways unavailable to haploids. In the same experiment, analysis of the relationship between ploidy and adaptation demonstrated that, overall, diploids adapted more slowly than haploids [57]. It was suggested that the slower adaptation of diploids observed in the evolution experiments in yeast grown on different media was caused by the reduced efficiency of selection for recessive or partially recessive beneficial mutations in diploid genomes [58, 59].

Johnson et al. [1] reported the results of another LTEE-similar study, in which 205 budding yeast populations (divided into haploid and diploid groups) have evolved under three different sets of laboratory conditions. The authors described the first 10,000 generations of yeast. They found that several aspects of evolution in this system were broadly consistent with findings from the LTEE and other long-term evolution studies. For example, the dynamics of fitness improvement was largely reproducible across replicate lineages and demonstrated a decline in adaptivity over time,

even while the rate of molecular evolution remained relatively constant. Evolution in diploid populations involved both fixation of heterozygous mutations and frequent occurrences of heterozygosity loss. However, there were key differences from the *E. coli* experiments, such as no evidence was found for a spontaneous emergence of stably coexisting lineages or for widespread evolution of mutator phenotypes resulting on sharp elevation of mutation rates, which could be due to a shorter time frame of the experiment, as well as a reduced indirect selection of mutators.

Johnson and Desai [60] developed an innovative yeast experimental evolution platform enabling insertion of barcoded deleterious mutations into the genome to measure their individual effects on fitness. This system made it possible to examine whether these mutations exert different effects on evolving lineages as these lineages continue to adapt over time. By applying the barcode-based mutagenesis system to analyze the adaptation effects of 91 specific mutations over 8000-10,000 generations of yeast in two constant media, the authors were able to describe how overall mutational tolerance of insertional mutations (defined based on the mean effect of this type of mutations), as well as adaptation effects of individual mutations, have changed throughout the course of evolution [60].

Analysis of the relationship between the organism size, metabolism, and demography in 12 *E. coli* populations evolving for more than 60,000 generations and diverging from a common ancestor [61] demonstrated that although experimental *E. coli* lineages that had evolved toward larger cell sizes exhibited relatively slower metabolism, they grew faster than the smaller cells. They achieve this growth-rate advantage by reducing relative costs associated with producing larger cells. Doubling cell size in experimental lineages resulted in only a ~30% metabolic increase. This observation is consistent with the results of other LTEE studies showing that evolved cells are larger, more efficient, and contribute to higher maximal biomass yields than their ancestors. It is worth noting that in the LTEE, larger evolved cells possessed slightly smaller genomes than smaller ancestral cells [14], which reduced relative and even absolute costs of genome replication. Most importantly, evolved cells have undergone significant adjustment of their gene regulatory networks to the LTEE environment, thereby reducing costly expression of unneeded transcripts and proteins [18, 19].

Experimental evolution studies have revealed a remarkable capacity of bacteria for a relatively rapid adaptation, which is often driven by mutations in central housekeeping genes responsible for fundamental cellular functions [62]. The most striking example of this trend involves mutations occurring in genes encoding the core enzyme RNA polymerase. It has been well established that the widespread use of antibiotics promotes accumulation and dissemination of resistant bacteria. However, resistance can also arise in the absence of antibiotic exposure. Antibiotic resistance mutations that arise in regulatory housekeeping genes, particularly, in RNA polymerase genes, may also exert adaptive effects independently of antibiotic presence, because they modify these genes in a way that eliminates the susceptibility to antibiotics. Many antibiotic resistance mutations are deleterious in antibiotic-free environments. Nevertheless, mutations that substantially alter an essential housekeeping gene may frequently carry additional fitness benefits unrelated to the antibiotic resistance. Adaptive mutations arising within antibiotic-targeted genes are often antagonistically pleiotropic (i.e., adaptive under certain conditions but deleterious under others). Such antibiotic-independent adaptive effects of resistance mutations can considerably alter the dynamics of the emergence and spread of antibiotic resistance. Understanding evolutionary pathways aimed at the resistance evolution in pathogenic bacteria is essential for developing effective strategies to combat infections.

It is important to emphasize that although these experiments have provided fundamental insights into evolutionary processes in controlled environments, evolution in nature occurs under much more complex conditions and may differ from the laboratory one. Under laboratory conditions, adaptation develops in response to simple and strong selective pressure; it also often results from mutations that are extremely unlikely to occur in nature. Cohen and Hershberg [63] studied two genes encoding *E. coli* RNA polymerase and found that under laboratory conditions, mutations occurred in highly conserved regions that evolve slowly in the wild and remain extremely conserved in their sequence, structure, and function from bacteria to humans. To determine whether this pattern is broadly applicable, the researchers examined 19 enzymes bearing adaptive mutations associated with the resource depletion and exposure to antibiotics. As with RNA polymerase, the loci of “laboratory” mutations were found to be highly conserved, frequently located within specific protein domains and in a closer proximity to the active site. Therefore, the dynamics of laboratory evolution differs markedly from that observed in nature. Selective pressure encountered in more natural environments is likely to be much more complex than the simple and strong pressure created under laboratory conditions, as in nature, multiple different factors exert conflicting pressures simultaneously and/or selective pressure alters over time. Consequently, adaptations that readily arise in laboratory experiments cannot be implemented as easily in the wild due to their pleiotropic effect. Moreover, positions that change effortlessly under a well-defined

selection in a laboratory are often among the least variable sites in nature. These observations suggest that mutations that are highly adaptive under a specific selective pressure may not contribute significantly to the overall adaptation in most natural environments or, if they do arise, are transient in natural bacterial populations [63].

P. anserina is a model ascomycete fungus that demonstrates pronounced phenotypic aging when grown on solid media, but exhibits an unlimited lifespan in a submerged culture. To investigate the genetic basis of adaptation to a submerged culture, *P. anserina* was established as a novel model system for LTEE. When grown in a submerged culture, most ascomycetes cease sexual and asexual reproduction and, instead, propagate vegetatively. Depending on particular cultivation conditions, the mycelium either grows dispersed or forms a spherical structure known as granuloma. In *P. anserina* populations, transition to long-term submerged cultivation invariably results in the same morphophysiological changes. These changes arise early in the experiment and are primarily attributed to epigenetic modifications that alter gene expression. The transition to an indefinite lifespan proceeds through three stages. Following transition to a submerged culture, non-adapted cells forms spherical cortical granules of darker color due to melanin synthesis. The granules then become smaller and begin to lose their dark pigmentation. Finally, in acclimated cultures, the mycelium becomes lightly pigmented and uniform, partly forming fluffy granules of varying sizes and partly remaining diffuse and structureless.

Two wild *P. anserina* strains were used to establish eight independent experimental populations, which were propagated by serial passaging and maintained in the dark in a standard synthetic medium for 8 years. With time, the number of single-nucleotide polymorphisms has linearly increased. Evolution in the eight experimental populations frequently proceeded in parallel, with the same genes and proteins experiencing emergence of the same mutations up to seven times. Six proteins associated with fungal growth and development had evolved in more than one population; notably, in seven out of eight populations, new alleles had been fixed in the FadA protein α-subunit gene, with only four amino acid sites affected, thus representing a unique parallelism in experimental evolution. Some of the six proteins undergoing parallel changes participate in the same pathways, and all proteins appeared to be associated with fungal growth and development, likely promoting vegetative growth and inhibiting sexual reproduction at a very early stage. Evolutionary parallelism at the protein function level was also observed for several transcription factors, suggesting selection leading to the optimization of a broad range of cellular processes under experimental conditions. Parallel evolution at the gene and pathway levels, excessive nonsense and missense substitutions, and increased conservation of proteins and their fragments affected by mutations suggest that many observed fixed mutations were adaptive and driven by positive selection [26, 33].

Laboratory evolution experiments have provided substantial insight into the relationship between the rates of genomic evolution and organism adaptation, genetic basis of fitness improvement under constant environmental conditions [12, 14, 64], morphological evolution of cells [64, 65], ecological specialization [53, 66], consequences of historical contingency and emergence of novel functions [2, 67], second-order evolutionary effects [68], and forces maintaining diversity [69]. They have enabled quantitative understanding of the correlation between the size, stability, and evolutionary potential of populations [70] and demonstrated that the most influential mutations are generally grouped into two classes – those that affect specific functions (e.g., in rate-limiting enzymes) and those that impair global transcriptional patterns (e.g., in RNA polymerase) [71]. The use of 12 *E. coli* lineages in the LTEE has enabled investigation of changes in the genotypes and phenotypes occurring in parallel in independently evolving bacterial populations [64, 68, 72]. The ALE technology has also been used to systematically define evolutionary mechanisms at the microbial metabolism and gene regulation levels [73]. Experimental evolution continues to expand its applications in biotechnology, in particular, for strain optimization in the production of novel compounds, increase of product titers, and enhancement of tolerance to adverse environmental conditions [74].

Initially employed to elucidate evolutionary mechanisms, ALE is now an important component of biotechnology-focused engineering strategies [75]. Being not limited by the requirements for the *a priori* understanding of genetic basis of target phenotypes, ALE has significantly expanded the potential for engineering non-model microbes, including those highly resistant to engineering. The molecular basis of adaptation, previously elusive, is becoming increasingly obvious due to advances in the high-throughput next-generation sequencing technologies. Table 1 summarizes selected ALE studies that have provided key insights into evolutionary processes, enabled widespread ALE application in metabolic engineering, and revealed molecular mechanisms underlying this strategy.

CONCLUSION

Evolution in a test tube has become a widely applied methodology in modern microbiology and

Table 1. Representative examples of results achieved using the ALE strategy

Species	Novel information obtained due to ALE implementation	References
S. cerevisiae, *E. coli*	on clonal interference and frequency dependence	[76, 77]
S. cerevisiae	on the evolutionary pressure and molecular mechanisms leading to the selection of multicellular forms of life	[42]
Candida albicans	on the dynamics and mechanisms of mutation development	[78]
S. cerevisiae	on the role of chromosomal rearrangements in the reversible cellular adaptation to a changing environment	[79]
E. coli	on the changing balance of forces driving genome evolution in populations adapting to a new environment, and genetic basis of enhanced fitness	[14]
E. coli	on the consequences of historical contingencies in laboratory evolution and emergence of a new function (aerobic citrate utilization or metabolism of the non-natural carbon source 1,2-propanediol)	[2, 67]
E. coli	on ecological specialization (high mutation rates as a driving force of specialization)	[66]
E. coli, *Gluconobacter oxydans*, *Torulaspora delbrueckii*, *S. cerevisiae*	on the molecular basis of microbial strain optimization through the enhanced utilization of substrate (glycerol, xylose, mannitol) or production of commercially valuable compounds (nonanedioic acid, ethanol, valencene)	[9, 10, 80, 81]
E. coli, *Propionibacterium acidipropionici*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, *S. cerevisiae*	on the molecular basis of microbial strain optimization through the enhanced stress tolerance (to isobutyl acetate, propionic acid, lactate, heat and osmotic shock, selenium, and furfural)	[82-87]

molecular biology. Some researchers use this approach to collect experimental data that can verify various evolutionary hypotheses and elucidate molecular basis of observed phenotypic changes. Others implement the methodological scheme of evolutionary experiments for applied studies aimed at selecting more productive and stress-resistant microbial strains required for modern industry [88]. At times, these directions intersect in an unexpected way. For example, *S. cerevisiae* strains exhibiting a distinctive snowflake phenotype had been originally obtained to address a solely fundamental question – to serve as a model for studying the emergence of multicellularity in eukaryotes [41]. Later, ALE was successfully applied to three non-flocculent industrial brewing strains of *S. cerevisiae* to obtain microorganisms with the aggregative (snowflake) phenotype, thereby demonstrating the use of ALE for improving the sedimentation properties of non-flocculent brewing strains [89].

To conclude, we have to emphasized three major issues. First, most ALE experiments are relatively short in duration – ranging from several weeks to a few months [16]. However, there are also long-term, multi-year projects that provide a much deeper understanding of evolutionary mechanisms and adaptation dynamics. Despite all their complexity, such studies reveal unique data on the sequence of mutations and molecular pathways underlying adaptive phenotypes – information that cannot be fully captured in short-term experiments [31]. One of the key advantages of laboratory evolution is its ability to explore the relationship between an increase in fitness and underlying mutations in evolved populations, at the same time solving the issue of interactions among single mutations. Compared with rational metabolic engineering, which directly introduces exogenous genes or disables endogenous ones, ALE cannot considerably alter metabolism within a short period of time.

However, the use of a rationally engineered strain as a starting point of evolution can substantially shorten the evolutionary process, particularly, when the acquisition of complex phenotypes is required [88].

Second, another important aspect is predictability of evolutionary trajectories. According to the research data, evolution may indeed be limited by a specific sequence of events, the so-called evolutionary pathway, in which certain mutations are more probable or advantageous at early stages than the others [27]. Nonetheless, it remains difficult to predict a full course of such trajectories, as they depend on numerous factors, including random mutations, selective forces, and interactions between mutations. Some studies demonstrate that early mutations can strongly influence subsequent evolutionary stages, providing the basis for predictive models; however, such approaches still require further development and validation.

Third, despite a significant progress achieved using ALE for both selection and investigation of complex phenotypes, several limitations remain, including those related to the population size, restricted time frames, simplified nature of laboratory environments, and possible misinterpretation of the roles of fixed mutations and selective forces [27, 90]. Therefore, when interpreting the results of experimental evolution, it is important to account for these limitations. Any prediction should be probabilistic and supported by additional experimental evidence.

Abbreviations

ALE	adaptive laboratory evolution
LTEE	long-term evolution experiment

Contributions

Ya.E.D. wrote the manuscript; O.A.K. collected and systematized the data; M.A.B. edited the manuscript.

Funding

This study was carried out as a part of the State Assignment to the Lomonosov Moscow State University.

Ethics approval and consent to participate

This work does not contain any studies involving human and animal subjects.

Conflict of interest

The authors of this work declare that they have no conflicts of interest.

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