Evolution in Microbes

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Abstract
This review presents a broad survey of experimental microbial evolution, covering diverse topics including trade-offs, epistasis, fluctuating conditions, spatial dynamics, cooperation, aging, and stochastic switching. Emphasis is placed on examples that highlight key conceptual points or address theoretical predictions. Experimental evolution is discussed from two points of view. First, population trajectories are described as adaptive walks on a fitness landscape, whose genetic structure can be probed by experiments. Second, populations are viewed from a physiological perspective, and their nongenetic heterogeneity is examined. Bringing together these two viewpoints remains a major challenge for the future.
INTRODUCTION

Microbes present our best chances to study the general principles of evolution. Short generation times, small genome sizes, and powerful genetic tools make for ideal organisms in laboratory evolution experiments. Microbes can serve as model systems to study most of the major aspects of evolution (37). Their vast species richness, physiological and behavioral sophistication, and intricate ecologies encompass a wide diversity of evolutionary questions.

Despite these advantages, the interpretation of microbial evolutionary experiments can often be difficult. Numerous studies have shown that, when given sufficient rounds of evolution, microbial populations eventually adapt to surmount diverse challenges. Beyond this nearly universal observation, quantitative prediction of most aspects of the adaptation process remains beyond our reach. For example, we cannot presently predict how long adaptation will take or in what form it will appear, how many mutations will occur, or how adapted the organisms will become. As in many complex systems, one does not expect to predict trajectories precisely, because of their intrinsically stochastic dynamics. Yet, with a theory in hand, one would like to predict correctly the distribution of outcomes. Theory should reveal the structure of dynamical laws that govern the adaptation process while enabling the construction of accurate, predictive models of real systems.

Do dynamical laws even exist in complex evolutionary systems? In recent experiments on closed microbial ecosystems, species’ densities were continuously observed over the timescale of months. Data collected on replicate populations demonstrated that their independent trajectories comprise a meaningful ensemble whose dynamical laws could be extracted by analyzing fluctuations (36).

Understanding how evolutionary dynamics arises from organismal physiologies and interactions poses a challenging open question. In this review, we introduce the major components of this puzzle and survey quantitative studies that could provide a basis for its solution. This review is meant to inspire many new investigations—both theoretical and experimental—by presenting a wide survey of topics.
EXPERIMENTAL EVOLUTION: EXAMPLES AND COUNTEREXAMPLES

Evolutionary experiments involve propagating populations over a large number of generations under controlled conditions (see Supplemental Figure 1; follow the Supplemental Material link from the Annual Reviews home page at http://www.annualreviews.org). Populations are assayed in time to determine changes in their composition. Comparisons with founder strains are made to quantify the overall degree of adaptation. DNA sequencing of specific genes or entire genomes is used to determine the mutational differences among evolved strains.

Although specific experiments differ in many respects, all seek to explain observed changes by developing appropriate models of organismal fitness. The concept of fitness is an abstraction, similar to the concept of energy in physics. As such, it comes in different flavors and is measured in different ways, summarized in Supplemental Figure 1. Fitness is a property of individual organisms; however, its measurement often involves large numbers of cells. For this reason, in any evolution experiment, a model of population dynamics must be used to infer fitness values from measurements. Fitness measurements are therefore limited by both experimental noise and model inaccuracy. In the following sections we discuss key evolution experiments, focusing on examples in which theoretical predictions were verified experimentally, and on counterexamples in which theory failed to explain or predict observations.

Surprises in Long-Term Evolution

In a famous and still-running experiment begun in 1988, 12 populations of Escherichia coli have been propagated by daily dilution into fresh media (50). In this long-term evolution experiment (LTEE), growth conditions consist of a single utilizable carbon source, glucose, which limits the maximal daily population size (Figure 1a). A single day consists of six or seven generations (i.e., cell doublings), followed by cells entering stationary phase until the next dilution. By now, this experiment has progressed for over 50,000 generations.

Whole genome sequences at six time points from the first 40,000 generations of a single population yielded some surprises (6). In the first 2,000 generations, this population’s average fitness increased by a factor of 1.5 (Figure 1b). In the next 18,000 generations, relative fitness in this population showed remarkable fluctuations, an outcome not anticipated by the theory (1). The population acquired a mutator phenotype around 26,500 generations, a phenomenon not predicted by the theory either (6). In the following section we discuss key evolution experiments, focusing on examples in which theoretical predictions were verified experimentally, and on counterexamples in which theory failed to explain or predict observations.

Fitness: a measure of an individual’s expected reproductive output and survival

LTEE: long-term evolution experiment conducted on 12 populations of Escherichia coli

Stationary phase: a physiological state that cells enter when nutrients have been exhausted

Figure 1

Major events and dynamics in the long-term evolution experiment (LTEE). (a) The LTEE on 12 populations of Escherichia coli, labeled Ara− (1–6) and Ara+ (1–6), has been running for over 50,000 generations (50). The time and population in which different adaptations occurred are indicated. (b) Dynamics of fitness (green squares) and mutations (blue circles) in a single population. The inset shows mutations on a different scale, as this population acquired a mutator phenotype around 26,500 generations. Panel b is adapted with permission from Reference 6.
fitness had rapidly improved by about 25%. Subsequently, the adaptation rate slowed down, and by 20,000 generations the population had only reached a 45% improvement (see Figure 1b). Genome sequences, however, revealed that the rate at which mutations became established was relatively constant, in seeming contradiction with the declining rate of adaptation. Neutral theory predicts a constant rate of establishment of new mutations, when these have negligible effects on fitness, yet several independent lines of evidence firmly rule out neutrality in these experiments. The observed behavior could be rationalized by assuming that the availability and fitness benefit of mutations are different in the early versus later parts of the experiment (see Fitness Landscapes and the Epistatic Genome, below).

Another surprise was the appearance of mutator phenotypes in five populations (51, 91). Earlier experiments had shown that mutators can outcompete nonmutator strains (15, 16). Nevertheless, spontaneous emergence of mutators in long-term evolution was largely unexpected, as mutators were thought to suffer a high deleterious mutational load. Theory suggests that mutators may be maintained at low levels within all populations and occasionally sweep to fixation with a beneficial mutation. Likewise, antimutators can subsequently outcompete mutators, leading to dramatic variations in mutation rate during the evolution of a single population (22). Such dynamics have not yet been observed, although the next 50,000 generations may hold new surprises.

While the 12 populations have been growing on glucose, the growth medium contained another carbon source, citrate, which these strains could not transport into the cell. Unexpectedly, one population evolved the ability to grow on citrate (denoted by \( \text{Cit}^+ \)) around 33,000 generations (10). This caused a dramatic increase in population size, because cells could continue to grow even after glucose had been depleted. Most interestingly, coexistence of \( \text{Cit}^+ \) and \( \text{Cit}^- \) cells has thereafter been maintained in this population. Upon dilution into fresh medium, \( \text{Cit}^- \) cells initially grow faster on glucose but plateau at lower density than \( \text{Cit}^+ \) cells. The constancy of daily fresh medium enables this long-term coexistence.

These experiments illustrate a breadth of evolutionary phenomena that can occur in even the simplest environments. To understand their causes, further studies have sought to characterize in detail evolutionary trajectories and fitness effects, which we now describe.

**Evolutionary Trajectories of Individual Mutations**

The dynamics of individual mutations are difficult to measure in general, because they initially arise spontaneously in single cells within huge populations. Once mutants reach appreciable frequency, dynamics can be tracked in various ways. For example, by mixing equal proportions of bacterial cells labeled with two different tags, beneficial mutation trajectories were observed in the final stages of their dynamics (35).

In yeast populations, clever experimental design allows earlier stages to be measured, starting from frequencies of 0.1% (47). This was achieved by constructing fluorescent markers for a general phenotype, sterility, which occurs frequently by mutation and confers a significant fitness advantage when cultures are propagated asexually. Automated liquid handling and daily measurements of marker frequencies allowed hundreds of populations to be propagated for 1,000 generations. From these trajectories, the initial fitness advantage of sterile mutants was measured to be 2% (on average). On their own, however, sterile mutations confer an average advantage of 0.6% or 1.5%, in two different genetic backgrounds that were used. This finding implies that within evolving populations, sterile mutants reach appreciable frequencies when they appear in already fitter backgrounds, i.e., in combination with other mutations. These experiments support recent theoretical analyses of adaptation in the presence of multiple mutations (21, 28, 31, 85).
Fitness Landscapes and the Epistatic Genome

Given that combinations of mutations, rather than mutations in isolation, are a major determinant of evolutionary dynamics, studies have probed the nature of interactions between mutations, also known as epistasis. These interactions are the basis for the structure of a fitness landscape, an abstract high-dimensional space in which genomes are viewed as nodes connected by edges corresponding to mutations. As populations evolve, they move across the landscape influenced by its fitness peaks and valleys. Direct analogies exist between populations dynamics on fitness landscapes and thermodynamics of physical ensembles on energy landscapes (20, 68).

To gauge the structure of fitness landscapes, strains carrying all possible combinations of several mutations have been genetically constructed, and their fitness was measured (17, 38, 83, 103). In one study, a single gene responsible for β-lactam antibiotic resistance in E. coli was mutated at up to five locations known to enhance resistance to the antibiotic cefotaxime, yielding $2^5 = 32$ different strains (103). The combination of all five mutations yielded a 10,000-fold increase in resistance. Among all possible mutational paths to attain these five mutations, it was found that 85% of paths were likely to be extremely rare as they included steps that decreased fitness or did not improve it (77). Different results were obtained in a second study, which investigated four mutations that arose in separate genes when Methylobacterium extorquens was evolved to employ a foreign pathway for growth on methanol (17). In this case, all mutational pathways were strictly uphill in fitness (i.e., the fitness landscape was smooth).

In these two studies, each mutation can exist in multiple backgrounds whose fitness had been measured (17). Hence it was possible to assess whether the fitness of the genetic background is at all predictive of the change in fitness due to each mutation. The methanol experiments revealed diminishing returns epistasis: It was found that the higher the background fitness, the lower the expected gain of each mutation. In contrast, for β-lactam adaptation, the effect of a mutation was not predictable on the basis of the background fitness. A third study examined in a similar way the first five mutations to fix in the LTEE (38) and found that four mutations exhibited diminishing returns while a fifth mutation exhibited synergy (i.e., the higher the background fitness, the larger the mutation’s benefit).

Dramatic consequences of epistasis were seen in experiments initiated from two different types of clones, EW (eventual winner) and EL (eventual loser), isolated from early stages of the LTEE (105). The EW clones had mutations that later became fixed in the population; however, early on these clones were less fit than EL clones by approximately 6%. Replicate evolution experiments started from each clone showed that, despite their initial fitness deficit, EW clones adapted much faster than EL clones, generating detectible beneficial mutants significantly earlier in most replicates. This behavior was traced to a difference between EW and EL clones in the DNA topoisomerase I gene (topA), which modulates DNA supercoiling with epistatic effects on many genes.

Theoretical analysis predicts how adaptation speed on fitness landscapes depends on the form of epistasis (45). When fitness landscapes exhibit antagonistic epistatic interactions, theory shows that although adaptation slows down, mutations still accrue at a nearly constant rate. On this basis, the data from the LTEE (see Figure 1b) were used to infer the structure of the epistatic landscape, which was consistent with strong antagonistic epistasis (45). Additional results on epistatic interactions are discussed in Reference 71.

Evolutionary Trade-Offs

Because microbes must survive under a large range of environments, natural selection may be faced with trade-offs: Improved performance in one set of conditions could adversely affect
Bacteriophage: a virus that infects bacteria

performance in another. Diverse evolutionary adaptations have been explained on the basis of trade-offs. Experiments in microbes allow several of these explanations to be tested.

The effect of temperature on growth rate has been observed in bacteria isolated from different natural environments, with different species displaying characteristic temperature-growth profiles (11). To investigate trade-offs, replicate populations of E. coli were grown at different temperatures (32°C, 37°C, or 42°C) for 2,000 generations and then transferred to growth at 20°C for an additional 2,000 generations (8). Fitness measurements showed that nearly all populations improved significantly in growth rate at 20°C, but the degree of improvement had no correlation with their ancestral growth temperature. The evolved lines were then assayed for growth at 40°C, with surprising results. Whereas a majority of populations exhibited a trade-off, one-third did not, and one population exhibited a statistically significant anti-trade-off, showing fitness gains at both temperatures. Moreover, the magnitude of fitness gains at 20°C exhibited no correlation with the magnitude of fitness losses at 40°C.

Experiments that probed trade-offs for growth on two different carbon sources revealed similar results (48): A trade-off was found in a majority of populations, yet one-fourth of populations exhibited an anti-trade-off, improving in growth on both media. Similar results were found for trade-offs related to aging in bacteria (3) (see Aging, below). From these experiments, it appears that the trade-off concept might be applicable only in some statistical sense—in any given experiment, the trade-off may or may not be apparent. While molecular constraints may impose an overall statistical trade-off on the adaptive landscape, a significant number of evolutionary paths might avoid trade-offs altogether.

The LTEE presents a beautiful illustration of the often counterintuitive nature of trade-offs. E. coli populations had evolved for 45,000 generations, adapting to growth on glucose in the absence of bacteriophages. Whereas the ancestral strain was susceptible to infection by λ phage, the evolved strains had developed complete resistance to it despite never encountering λ phage in their evolution (63). The reason for this unexpected result was illuminating. λ Phage infects E. coli through LamB, an outer membrane receptor that is used to import maltose into the cell. Adaptation to growth on glucose involved mutations that turned off the maltose pathway and its receptor, which subsequently led to λ phage resistance. Many additional examples regarding trade-offs and optimality are seen in evolutionary studies of bacteriophage T7 (12).

Adaptation to Fluctuating Environments

Evolutionary trade-offs are most relevant when adaptation occurs under fluctuating environments. Nutrient fluctuations, for example, are one large class of environmental changes to which bacteria have adapted. Expression and regulation of sugar operons, such as the lac operon, allow bacteria to grow on carbon sources other than glucose. When conditions change, for example when glucose is depleted and lactose becomes available, cells express the lac operon, producing the permease LacY, which imports lactose into the cell, and the β-galactosidase LacZ, which hydrolyzes the lactose molecule. In the absence of lactose, the operon is repressed by the repressor Lacl to alleviate the cost of expression (23).

Experiments have begun to probe how regulation evolves under fluctuations. A recent study followed 2,000 generations of E. coli populations growing under four different regimes, both constant and fluctuating: (a) constant glucose, (b) constant lactose, (c) constant glucose and lactose, and (d) daily alternation of glucose and lactose (79). The evolved strains exhibited a variety of changes in the lac operon, from lower induction thresholds, to loss of bimodality, to complete loss of regulation. Different mutations in lacI were observed, as well as mutations in genes with no
previously known role in lactose metabolism. Interestingly, populations that grew in the alternating environment typically evolved to constitutively express the operon, losing regulation.

Understanding trade-offs is key to explaining how a lack of regulation can be selectively advantageous in fluctuating conditions. To this end, another recent study employed an engineered operon under the control of the lac promoter, which confers a cost or a benefit under two conditions (76). Expression of the operon provides resistance to the antibiotic chloramphenicol (cm) but leads to a toxic effect in the presence of sucrose. By artificially inducing the operon to different expression levels, it was possible to sensitively quantify growth rates $F_{\text{cm}}$ and $F_{\text{sucrose}}$ under the two conditions (see Figure 2). As expression levels are changed, the growth rates trace out a curve in the $F_{\text{sucrose}}$–$F_{\text{cm}}$ plane. The shape of these curves, their concavity or convexity, depends sensitively on the concentrations of sucrose and chloramphenicol. And, importantly, the shape determines whether the optimal expression level in fluctuating conditions will occur at an intermediate level or at one of the extremes, which correspond to loss of regulation. These measurements formed

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**Figure 2**

Trade-offs due to varying gene expression in two different environments (76). (Left) Growth rates of *Escherichia coli* were measured at different levels of induction of the engineered operon, which confers both resistance to chloramphenicol (cm) (top) and toxicity in the presence of sucrose (bottom). (Right) The measurements, replotted on the two growth-rate axes shown, yielded curves of different concavities. Gray lines correspond to isoclines of the average growth rate when environments alternate for equal periods of time. Arrows indicate the position of the optimal expression level, which is seen to be intermediate for the red curve and extreme for the blue curve. Adapted with permission from Reference 76.
a basis for highly successful selection experiments, in which an anti-Lac repressor (with inverted regulatory behavior) was evolved in three rounds of mutagenesis and selection (76).

Spatial Dynamics

Evolutionary dynamics can differ substantially between well-mixed populations and spatially distributed or otherwise subdivided populations. In well-mixed populations, resources are global and the rise of a beneficial mutation in a subpopulation can rapidly be felt by the rest of the population. In subdivided populations, resources are local and individuals are in direct competition only with their immediate neighbors. If a beneficial mutation arises in one part of the population, the rest of the population may not feel any effect for a long time, because local resources remain unaffected. This increases the time to fixation for beneficial mutations while allowing the population to maintain more genetic diversity.

To test whether the greater diversity afforded by subdivision could be beneficial, separate populations of yeast cells were propagated and periodically mixed (44). After 550 generations of evolution at different migration rates between populations, fitness measurements showed that well-mixed populations were consistently fitter than subdivided populations. It was concluded that the fitness landscape in these experiments must be relatively smooth, favoring fast fixation of beneficial mutations instead of more thorough exploration of the landscape.

Spatial trajectories of microbial evolution have been intensively studied over the past five years, both theoretically and experimentally (32, 33, 41–43, 70). The experimental model consists of microbial colonies of either bacteria or yeast cells inoculated on nutrient-rich plates and grown over several days. The initial inoculation contains a 50:50 mixture of two strains differing only in a neutral fluorescent marker. After growth, the plates are imaged and the spatial pattern of the markers is analyzed.

Visually arresting patterns of spatially segregated domains of each marker appear, after an initial growth period, and continue to propagate in time (see Figure 3a). This behavior, known

![Figure 3](image)

**Figure 3**

Dynamics of spatial growth. (a) Two identical strains of *Escherichia coli*, fluorescently labeled green or red, were inoculated at the center. Their growth leads eventually to segregated domains at the colony edge, at which only a single strain (color) is present. (b) Beneficial mutations are seen via emergence of sectors. (Top) A green sector emerged spontaneously in a growing yeast colony consisting of a 5:1 mixture of red:green cells. (Bottom) Sectors are seen when a strain with a known beneficial mutation (red) was mixed in a 1:40 mutant-to-wild type ratio. Adapted with permission from Reference 33.
as genetic demixing, occurs because of random motion and reproduction at the edge of a growing colony (32, 70). Physical models reveal that although demixing occurs at the colony boundary, the expansion of the boundary allows diversity to be maintained indefinitely (33, 41). The finite number of domains maintained can be computed exactly, and its predicted dependence on the size of the initial inoculation has been verified by experiments using two different bacterial species (43).

Growth patterns of *E. coli* on plates have a chiral component, in which the entire population twists counterclockwise when viewed from below the plate. The effect of this chirality was subtracted off and the remaining motion of boundaries was seen to be diffusive (43).

In the presence of selective differences between strains, spatial growth patterns exhibit characteristic shapes, such as bulges, at the colony edge (Figure 3b). The boundary of these bulges takes the form of logarithmic spirals, a theoretical prediction that was verified in experiments using yeast cells (42). A labeled sterile yeast mutant was mixed at a low starting frequency with wild-type cells. When sterile sectors appeared, their opening angles and shapes allowed estimates of their fitness advantage to be made.

These experiments reveal at a new level of detail how selection and drift are influenced by spatial structure, as well as the essential components of their theoretical description. At the same time, they provide new tools for measuring selective differences between competing lineages.

**INTERLUDE**

By this point, thanks to the author’s biased selection of examples and counterexamples, the reader may be forming some opinions. On the one hand, examples of predictions verified by experiment have been presented. On the other hand, counterexamples in which key experiments negate theoretical expectations, or yield wholly unexpected results, have been discussed. Individual opinions on this state of affairs will vary and may include the following:

1. There is no problem with theory—evolutionary experiments are simply not sufficiently well controlled.
2. There is no problem with experiments—theory is not (yet) sufficiently complex to account for all the phenomena.
3. There is no problem at all—for every observed evolutionary phenomenon, there already exists a theory to explain its behavior.
4. There is a big problem—we have no real theory, and therefore we do not know which experiments to do.

One feature of purely mathematical models of evolutionary dynamics is that they abstract away physiology, replacing it with a collection of selective coefficients and a fitness landscape. Such abstraction would appear to be a good thing: One would like to describe universal aspects of evolution and ignore the messy details of biology. Physical theory is particularly good at such abstraction. The equations of electromagnetism, for example, make no mention of details such as wires, light bulbs, or iPads. Yet the striking advantage of physical theory is that one knows how to put the details back in. Indeed, Maxwell’s equations provide the ultimate guide for deriving explicit, predictive models of real systems. In mathematical evolutionary theory, abstraction is often a one-way street: We do not know how to put the details back in.

Are there more physical routes to abstraction in evolutionary theory? In the second half of this review, we consider physiological aspects of microbial evolution as a starting point to inspire new theoretical directions.
QUANTITATIVE PHYSIOLOGY OF MICROBES

The quantitative basis of experimental evolution lies in the complex dependence of growth rates on physiological states and external conditions. We survey major facets of microbial physiology that are critical for evolutionary experiments, focusing on recent quantitative studies with relevant theoretical aspects.

Exponential Growth

Exponential growth is achieved by bacterial populations when nutrients are at sufficiently high concentrations while cell densities remain sufficiently low. Such growth can be maintained in chemostats, turbidostats, and microfluidics devices. Monod’s experiments yielded a simple relationship between steady-state growth rate, λ, and the concentration of a limiting nutrient, N:

\[ \lambda = \frac{\lambda_{\text{max}} N}{N + K}, \]

where \( \lambda_{\text{max}} \) and \( K \) are parameters that depend on the specific nutrient as well as the growth medium (65). One rationalizes Monod’s law by imagining bacterial cells as nutrient absorbers that undergo simple molecular binding at specific receptors. Internal enzymes convert nutrients into cellular biomass, which in simple cases may involve a single bottleneck reaction. The biomass flux is then determined by Michaelis-Menten kinetics, which leads to Monod’s law.

The simple form of this growth law hides much complexity. How do the parameters \( \lambda_{\text{max}} \) and \( K \) depend on the internal state of the cell? What are the molecular determinants of the maximal growth rate, and how is it established? These questions were recently addressed on the basis of two general observations related to the (mass) ratio of cellular RNA to protein, \( r \) (see Figure 4):

(a) The ratio \( r \) exhibits strong positive correlation with the growth rate \( \lambda \) when nutrient quality is varied (87). (b) The ratio \( r \) exhibits strong negative correlation with \( \lambda \) when protein translation rates are varied, via antibiotics or genetic mutations (88).

Figure 4

Model of steady-state exponential cell growth and measurements under different nutrient and antibiotic conditions. (a) Ohm’s law can be applied to describe exponentially growing cells (88). (b,c) Symbol shapes and colors correspond to a wide variety of nutrient conditions. In panel b, plot points correspond to different growth media; in panel c, plot points correspond to increasing chloramphenicol levels in different media (88). The (mass) ratio of RNA to protein, \( r \), is directly proportional to \( \phi_R \), the fraction of the proteome dedicated to ribosomes. Because ribosomal RNA constitutes the dominant fraction of all bacterial RNA, stoichiometric relations dictate that \( \phi_R = \rho r \), where \( \rho \) is estimated to be \( \approx 0.76 \). Adapted with permission from Reference 88.
By analogy with Ohm’s law, then, which was measured by Scott et al. (88) to be the negative correlation of we increase the translational capacity, the voltage across the ribosomal resistor drops, revealing because growth on a limiting nutrient involves bottleneck enzymatic reactions, the flexible fraction, where metabolic enzymes reside, would adjust to the nutritional demands of the growth media. Because growth on a limiting nutrient involves bottleneck enzymatic reactions, $dM/dt$ is proportional to the number of enzymes, scaling as $\phi_P \cdot M$. This yields the exponential growth rate $\lambda = \kappa_s \phi_P$, where $\kappa_s$ is the nutritional capacity, a parameter determined by enzymatic rates, nutrient concentrations, and medium composition.

The two different expressions obtained for $\lambda$ are subject to the constraint $\phi_R + \phi_P = \phi_R^{\text{max}}$. By analogy with Ohm’s law, then, $\phi_R$ and $\phi_P$ act as voltages across resistors wired in series, with conductance $\kappa_R$ and $\kappa_s$, respectively; and $\lambda$ corresponds to the net current through the circuit (see Figure 4). If we increase the nutritional capacity, we increase the net current, hence the voltage $\phi_R$ across the ribosomal resistor increases. This reveals the positive correlation of $\phi_R$ and $\lambda$ when growth rates are manipulated by varying the medium composition. Alternatively, if we increase the translational capacity, the voltage across the ribosomal resistor drops, revealing the negative correlation of $\lambda$ and $\phi_R$ when translation rates are manipulated. Expressing the net current explicitly yields

$$\lambda = \frac{\phi_R^{\text{max}} \kappa_s \kappa_R}{\kappa_R + \kappa_s},$$

which is a reformulation of Monod’s law by quite different reasoning, and with the more general quantity $\kappa_s$ in place of nutrient concentration.

This elegant formulation could provide a conceptual and quantitative basis to describe evolution during steady-state growth. Mutations may independently alter translational and nutritional capacities, $\kappa_R$ and $\kappa_s$, as well the cell’s growth potential $\phi_R^{\text{max}}$; or, they may simultaneously affect combinations of these complex parameters. The basic ideas on the logic underlying regulation of proteome partitions have been discussed (39) and could form a theoretical basis for prediction. Generalization for growth under fluctuating conditions, away from the steady state, is an open avenue for research.

**Physiological Heterogeneity**

Microbial populations are known to be heterogeneous. Cells within the population exhibit a range of internal states, or phenotypes, that influence their physiological behaviors. Phenotypic states are determined by many factors, including expression levels and activities of genes, methylation states of promoters, concentrations of nutrients, and mechanical properties of cells. Cells are able to switch between phenotypic states, which can strongly influence their reproduction and survival. Under stressful conditions, for example, such as high temperatures or starvation, bacteria activate stress response pathways, which protect cells and increase survival rates.
When cells divide, their phenotypic states can be passed on to their progeny. The stability of states—their heritability—can vary dramatically among phenotypes. Phenotypes that are coded directly by DNA, e.g., the allelic states of genes, are the most heritable because stability is limited only by the mutation rate ($\sim 10^{-10}$ per base pair per division in *E. coli*). However, certain DNA sequences (e.g., simple repeat sequences) are hypervariable. Such repeats can mutate at rates spanning orders of magnitude, from $10^{-8}$ to $10^{-4}$ per cell division, and are observed to vary between different bacterial strains (57). Repeats in promoters and coding regions can lead to spontaneous phenotypic switching and maintenance of heritable heterogeneity (66, 98). Even nongenetically determined phenotypes, such as the induction state of the *lac* operon (97), or methylation states of promoters (56), can be stably inherited for hundreds of generations.

Timescales of heritable physiological variation within microbial populations span a wide continuum (80). Phenotypic variation can develop quickly within populations, indeed, much faster than genetic variation, and can be beneficial. For example, slow growth appears to be a generally protective state. Measurements in yeast cells have shown that genetically identical cells exhibit a wide distribution of growth rates, and that slow-growing cells exhibit increased tolerance against stress such as heat killing (55). The existence of heritable physiological variation could strongly influence the outcome of evolution experiments (see A Final Example, below). Several well-characterized examples of physiological heterogeneity are discussed in the following section.

**Stochastic Switches**

Stochastic switches are molecular mechanisms that allow cells to spontaneously switch their phenotypic states without the use of sensory responsive pathways. Such switches have been studied widely in the field of pathogenic bacteria, where they are often involved in evading host immune responses (66, 96). Quantitative analysis of stochastic switches has advanced rapidly in recent years through single-cell microscopy and microfluidics-based studies (59). Closely related theoretical works have analyzed the benefits of switches in fluctuating environments (46, 95, 104). In particular, these studies predict that the long-term growth rate of stochastic switching bacteria will depend sensitively on their switching rates, hence the rates themselves could be evolutionarily tuned.

The persistence switch in *E. coli* is a stochastic switch that maintains a subpopulation of slow-growing persister cells at frequencies of $10^{-6}$ in wild-type strains (5). Its molecular mechanism was recently elucidated (84). Persister cells are highly tolerant of certain antibiotics and other stresses and enable populations to survive in conditions of fluctuating antibiotics (54) or bacteriophages (75). Another well-characterized stochastic switch of evolutionary significance is the competence switch of *Bacillus subtilis*, which activates a phenotypic state that allows cells to take up DNA from their environment (93). When starved, cells stochastically choose between activating the competence state and turning on sporulation genes to begin a developmental program whose completion results in a highly resistant spore.

In a few cases, competition experiments have been conducted using strains with different switching rates. In the case of persistence, two strains were used—wild type and the *bipA7* high-persistence strain (5). A more complete set of competitions was performed using a synthetic stochastic switch constructed in yeast cells (1). In these experiments, performed in turbidostats, fast-switching strains outcompeted slow-switching strains when environmental fluctuations were rapid, whereas the reverse was seen when fluctuations were slow, consistent with theoretical predictions.

These studies suggested that it may be possible to evolve stochastic switches in the lab, by exposing populations to fluctuating selection. Evolution of a reversible stochastic switch was achieved using *Pseudomonas fluorescens*, which exhibits different niche-specialized phenotypes with
altered colony morphologies. By directly selecting for novel colony morphology, picking individual colonies, and propagating over multiple rounds, eventually a strain emerged that stochastically switched between opaque and translucent colonies (7). The ability to switch was traced to a single nucleotide change in the gene \textit{carB}, which is involved in arginine and pyrimidine biosynthesis. Its role in switching has not yet been revealed.

Stochastic switching rates are typically much larger than mutation rates, yet much smaller than cell division rates. Hence, they yield heritable phenotypic variation within populations, which selection can act upon, with potentially large effects on evolutionary outcomes. How can we detect their presence within microbial populations? Recent theoretical analysis has shown that by measuring the variance of fitness between single-cell histories, one can detect stochastic switches. This analysis is based on a path-integral formulation of populations in which the history of each cell (and its ancestors) constitutes a set of trajectories (49). Cell divisions observed along each history can be used to infer the fitness of cells and assign a fitness to each history. Large variance of this historical fitness across the population is a sensitive detector of stochastic switching events.

The description of populations as thermodynamic ensembles of individual histories can also be generalized to include the effects of aging and cell cycles (99), as discussed in the following section.

\section*{Aging}
Aging, in the evolutionary context, is the reduction in average reproductive output as a function of an individual’s age. In microbes, aging has been studied in both yeast and bacteria (see, e.g., 4, 90). Numerous molecular mechanisms underlie aging in microbes, including accumulation of misfolded aggregated proteins, toxic compounds, and other damage to cellular components that is not efficiently repaired. Upon cell division, damage is partitioned between the two cells.

Damage repair and asymmetric damage partitioning are two strategies that cells use to counteract damage accumulation. Both require investment of energy. For repair processes, ATP-dependent chaperones help refold misfolded proteins, and degradation and new synthesis are used for damaged cellular components such as membranes. Asymmetric partitioning likewise requires ATP-dependent transport of aggregates, such that after cell division one cell will carry less damage; that is, it will be rejuvenated relative to the other cell (82).

How much energy should cells invest in repair versus asymmetric partitioning? Naively, the question appears to involve accounting for energy of transport versus repair, with the simple outcome that the cheaper process would be preferred. However, these two strategies reap their benefits in very different ways. Efficient repair maintains all cells at some mean damage level, reducing cell-to-cell fluctuations. Asymmetric damage partitioning increases cell-to-cell variability by rejuvenating only one of the two cells at each division. Within an actively dividing population, selection can act to dilute out the cells that carry more damage, as these cells divide slower than cells with less damage.

Simple models have been used to explore how the overall shape of the aging profiles and repair parameters influence the degree to which asymmetry is evolutionarily favored (2, 24). Applying conclusions from models to experiments is a much more delicate problem. For example, evolutionary models of aging often assume the existence of trade-offs, such that faster reproduction rate at young age comes at the expense of slower growth at older ages. A laboratory evolution experiment in which strong selection was applied to favor mutations that increase growth rate in young cells, however, yielded mutants in which even very old cells grew significantly faster than in the original strain (3).

Detailed measurements of bacterial aging have become possible by innovative applications of microfluidics and microscopy. By continuously imaging cell divisions over multiday periods, it
was found that *E. coli* cells have the ability to maintain uniform growth rates for at least 100 cell divisions (100). After approximately 30 divisions, however, cell mortality becomes a significant factor that affects an increasing proportion of cells. Interestingly, in the first 8–10 cell divisions, a small reduction in growth rate was seen in different experiments (58, 92, 100), an observation interpreted as evidence of cellular aging (92), physiological adaptation (100), or trade-offs between damage and segregation processes (14, 81).

The interplay between cellular energetics, physiological variability, and selection within populations lies at the heart of these experiments. To elucidate its underlying principles, a statistical mechanical approach has been formulated (99): Single-cell histories make up a space of conformations (or trajectories), selection acting on histories behaves analogously to energy, and the intrinsic variability in the timing of cell divisions results in entropy. Optimal lineages, histories that optimize a trade-off between selection and entropy, determine the population’s growth rate, a free-energy-like quantity. The strength of selection acting at different ages can be directly determined from the distribution of ages along optimal lineages. Understanding how cellular thermodynamics influence, and are influenced by, the thermodynamics of lineages poses another challenging theoretical question.

**Collective Physiology: Quorums, Swarms, and Biofilms**

The coexistence of different cellular phenotypes within populations provides opportunities for additional physiological behaviors, mediated by interactions between cells. These behaviors can be cooperative, if cells engage in collective activities, such as swarming, creating biofilms, or producing light; or specialized, if cells are capable of better exploiting certain metabolites, such as the citrate phenotypes discussed above; or antagonistic, for example, when cells produce toxins to kill competitors or when cells cannibalize their neighbors (86). Theoretical treatment of dynamics of interacting cells has revealed a rich range of new behaviors, which can often be described in game-theoretical terms (19, 26). In this section, we focus on collective behaviors, which are often regulated by or subject to the overall density of the population.

**Cooperation and cheating.** When cells appear to cooperate, the potential for cheating is always present. Cells that do not pay the physiological costs associated with a collective activity can often profit from the investments of neighboring productive cells. For example, to metabolize sucrose, yeast cells produce the enzyme invertase, which hydrolyzes the disaccharide into glucose and fructose. Because the reaction occurs outside the cytoplasm, the monosaccharides can diffuse away, becoming a public good available to other cells. Experiments using cheater mutants that do not produce invertase show that nonproducers have a competitive advantage in the presence of producers (29). Conversely, when producers are a minority, they increase in numbers against the cheaters. A stable coexistence between the two strains is rapidly attained. In the presence of excess glucose, however, producers lose any advantage and can be driven to extinction.

How are cooperative interactions sustained in the presence of cheaters? If conditions are right, population subdivision can enhance cooperation. Although cheaters outcompete cooperators within each subpopulation, the overall growth of different subpopulations is enhanced by their fraction of cooperators. In a regime of periodic mixing and subdivision, it is possible for cooperators to increase in the population at large while their fractions decrease within each subpopulation. This scenario, known as Simpson’s paradox, was recently constructed in a synthetic microbial system (18), which used quorum-sensing autoinducer molecules as a public good.
BEYOND PHYSIOLOGY: ECOLOGIES, INSIDE AND OUT

Metagenomics analyses are dramatically revealing the ecological structures of bacterial communities (27). These analyses have uncovered a prevalence of horizontal gene transfer in bacteria much higher than previously thought; hence purely asexual evolutionary dynamics are an inadequate representation of real bacterial evolution (25, 53, 89) and models incorporating recombination must be considered (72). Bioinformatic analyses suggest that horizontal transfer can strongly influence the evolution of cooperation in bacteria (74). Studying ecological interactions in quantitative laboratory experiments is a major frontier for future research (36).

An entirely different ecology exists in microbes—an ecology of mobile genetic elements, such as transposons, phages, and plasmids, that can move around within genomes and between cells. Their influence on adaptation is highly significant, as seen in studies of yeast adapting to nutrient-limited chemostats (30). The evolutionary pressures that mobile genetic elements experience and exert on their hosts are highly nontrivial (13). Several striking examples involve toxin-antitoxin modules in bacteria (108) and restriction-modification systems (40, 78). The coevolution of phage and bacteria presents an additional set of challenges regarding self-nonself recognition and immunity (67). Recently discovered CRISPR systems in bacteria (61), which provide immunity against phage as well as memory of past infections, have inspired new theoretical works (52, 102). The world of bacterial mobile elements overflows with fascinating evolutionary complexity.

Quorums. Population density is sensed by bacteria using genetic pathways known as quorum-sensing networks. These networks include genes for production, export, and sensing of small-molecule signals, known as autoinducers. Using specific receptors that bind autoinducers, quorum-sensing networks measure concentrations and respond at specific autoinducer levels to regulate downstream genes. Quorum sensing was first discovered in connection with a specialized organ of a Hawaiian squid that harbors a population of light-emitting *Vibrio fischeri* bacteria (73, 101). Because light production is beneficial to the squid host only when a large number of bacteria are present, the bacteria use quorum sensing to activate the behavior only at high density, saving the cost of light production when cells are at lower density.

Bacteria typically express more than one quorum-sensing system, each specialized for a given autoinducer molecule. Information from multiple autoinducer signals is integrated by cells and used to make internal decisions regarding which sets of genes to activate or repress (60, 94). For example, *Vibrio harveyi* uses three different autoinducers: One is produced by a large number of bacterial species, another may be specific only to *Vibrio* bacteria, and the third is specific to the *V. harveyi* species. Intriguingly, numerous examples of interspecies molecular warfare have been described in which one species specifically destroys the quorum-signaling molecules of another (a process named quorum quenching).

What is the evolutionary logic underlying integration of quorum-sensing signals? What are the meanings that evolution has devised for different signaling molecules in bacteria? These questions remain under intensive investigation, using concepts from communication theory (9, 62). Beyond their evolutionary logic, the presence of quorum-sensing networks in bacteria has immediate implications for experimental evolution. When cell densities remain constant (e.g., in chemostats or turbidostats), there may be no pressure to maintain these systems. However, when densities change dramatically over time (e.g., for experiments in batch culture or on plates), fitness landscapes may be modulated by density-dependent factors mediated through quorum-sensing systems, which can affect hundreds of genes.
Swarms. In collective behaviors, the connection between individual physiological costs and fitness can be particularly subtle. Swarming motility in the bacterium Pseudomonas aeruginosa provides a key example. In order to swarm, P. aeruginosa cells produce and secrete large amounts of rhamnolipid biosurfactants, preparing a wet surface on which the collective motion can take place. Nonproducer cheater mutants, deficient in rhamnolipid production, are fully capable of swarming on the secretions of wild-type cells (see Figure 5).

Because the physiological cost of rhamnolipid production is high, it was expected that cheaters would have an advantage swarming when mixed with wild-type cells. However, after passaging mixtures of the two cell types for multiple days on swarming plates, no fitness cost was seen—the two populations were maintained in constant proportions (107). Much further analysis revealed that the production rate of rhamnolipids, though regulated by quorum sensing, is ultimately determined by growth rate rather than density. When essential nutrients for cell growth (e.g., nitrogen) are depleted, cells turn on rhamnolipid production, using excess carbon to build the biosurfactants.

The physiological cost of production therefore does not translate into a fitness cost, due to this metabolically prudent behavior (107): Production is regulated to occur under conditions in which cells are not actively growing. For this reason, although they can be maintained, cheater mutants cannot invade the wild-type population. Just how these cells manage to integrate quorum-sensing signals with other signals to produce a precise measurement of their own growth rate remains a tantalizing question.

Biofilms. Bacteria growing on surfaces are capable of forming dense, structured communities known as biofilms. To establish a biofilm, many species produce and secrete extracellular polymeric substances (EPS), which form an extracellular matrix structure supporting the cells. As with rhamnolipid synthesis in the case of swarming, EPS production involves fitness costs that could be subject to cheating by nonproducers. Recent experiments using mixtures of producers and nonproducers demonstrate fitness costs of EPS production in liquid culture—i.e., in the absence of...
actual biofilm structure—while revealing that producers rapidly outcompete nonproducers when growing within biofilms (69).

Remarkably, this behavior was previously predicted by detailed simulations of biofilms (106). Owing to spatial effects, the EPS structures themselves promoted the growth of producer cells through formation of tower-like structures, very similar to the actual structures observed with confocal microscopy (69). This enables producers to reach higher nutrient concentrations while suffocating their nonproducing neighbors. Rather than favoring nonproducing cheaters, competitions within biofilms may select for vigorous, efficient EPS production. Interestingly, experiments using mixed-species biofilms have found that certain species exhibit specialized phenotypes, adapted for biofilm growth in the presence of competitors, that can increase in frequency by selection (34).

A FINAL EXAMPLE

This review began with a series of key examples from evolutionary experiments and then shifted to quantitative microbial physiology, surveying cellular processes that determine growth rates and form the basis for evolutionary selection.

A final example illustrates how physiological effects conspire to dramatically affect evolution. Populations of E. coli were coevolved with λ phage in a glucose-limited environment for 28 days (64). Very quickly, within 8 days, mutants that downregulated the LamB outer membrane receptor, which the phage uses to infect cells, became fixed in the population. Notwithstanding this calamity for the phage, a tiny population of phage was still maintained after 8 days. These phages were growing on a small subpopulation of E. coli that stochastically expresses the LamB receptor. Remarkably, sometime between 8 and 17 days, the phage population evolved an innovation: the new ability to infect through OmpF, a different outer membrane receptor. Thus, the propagation of phage on a tiny subpopulation of E. coli maintained by stochastic switching enabled a major evolutionary innovation. And this was only half the story (64).

In summary, elucidating the relationships between evolutionary dynamics and microbial physiology presents a series of worthy challenges for many future studies. Undoubtedly, achieving this goal will require the critical input of many different approaches across diverse fields and disciplines.

SUMMARY POINTS

1. Evolutionary experiments in microbes are probing the structure of fitness landscapes, testing quantitative predictions of adaptation rates and spatial dynamics.

2. Microbial physiology provides the mechanistic basis for growth rate differences on which evolutionary selection acts.

3. Understanding quantitatively how selection and physiology interact remains a major challenge for future studies.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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