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REVIEW

Experimental Design, Population Dynamics, and Diversity in Microbial Experimental Evolution

Bram Van den Bergh,[a,b,c] Toon Swings,[a,b] Maarten Fauvart,[a,b,d] Jan Michiels[a,b]

[a]Laboratory of Symbiotic and Pathogenic Interactions, Centre of Microbial and Plant Genetics, KU Leuven-University of Leuven, Leuven, Belgium

[b]Michiels Lab, Center for Microbiology, VIB, Leuven, Belgium

[c]Douglas Lab, Department of Entomology, Cornell University, Ithaca, New York, USA

[d]imec, Leuven, Belgium

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Address correspondence to Bram Van den Bergh, bram.vandenbergh@kuleuven.vib.be, or Jan Michiels, jan.michiels@kuleuven.vib.be.

B.V.D.B. and T.S. contributed equally.

SUMMARY In experimental evolution, laboratory-controlled conditions select for the adaptation of species, which can be monitored in real time. Despite the current popularity of such experiments, nature's most pervasive biological force was long believed to be observable only on time scales that transcend a researcher's life-span, and studying evolution by natural selection was therefore carried out solely by comparative means. Eventually, microorganisms' propensity for fast evolutionary changes proved us wrong, displaying strong evolutionary adaptations over a limited time, nowadays massively exploited in laboratory evolution experiments. Here, we formulate a guide to experimental evolution with microorganisms, explaining experimental design and discussing evolutionary dynamics and outcomes and how it is used to assess ecoevolutionary theories, improve industrially important traits, and untangle complex phenotypes. Specifically, we give a comprehensive overview of the setups used in experimental evolution. Additionally, we address population dynamics and genetic or phenotypic diversity during evolution experiments and expand upon contributing factors, such as epistasis and the consequences of (a)sexual reproduction. Dynamics and outcomes of evolution are most profoundly affected by the spatiotemporal nature of the selective environment, where changing environments might lead to generalists and structured environments could foster diversity, aided by, for example, clonal interference and negative frequency-dependent selection. We conclude with future perspectives, with an emphasis on possibilities offered by fast-paced technological progress. This work is meant to serve as an introduction to those new to the field of experimental evolution, as a guide to the budding experimentalist, and as a reference work to the seasoned expert.

KEYWORDS adaptive evolution, evolution experiments, evolutionary biology, experimental evolution, microbial ecology

INTRODUCTION

Ever since Darwin published his seminal work on evolution by natural selection that acts on diversity and selects the most fit individual (1), there has been great interest in understanding evolution and its underlying principles. According to Darwin's theory and with his focus on large, higher eukaryotic species, it was thought that evolution was too slow to be studied directly and that it could be done only by indirect comparisons of living species and/or fossils. Such comparative studies are still of great value today, especially when looking at long time scales and when aided by modern techniques to determine and compare, for example, sequences of DNA or proteins (2–4). Nowadays, however, it has become clear that for many organisms, especially for microorganisms, evolutionary changes can also happen over shorter time periods. Rearing species in controlled environments for an extended time thus allows the monitoring of evolutionary adaptation in real time and has opened a new, broad field of research (5). In retrospect, the field of experimental evolution was actually already born as soon as William Dallinger, a contemporary of Darwin, showed that protozoa could be selected over time to grow at extreme temperatures (6). Sadly, Dallinger was too far ahead of his time. Darwin found his observations curious and interesting, and Dallinger received appraisal from many of his peers, yet the new domain of science was left in its infancy for a long time.

Initially, evolutionary studies focused on higher eukaryotic species, likely because of

the limited knowledge on microorganisms and their underrated biological relevance. Here, experimental evolution can be useful to study the evolution of multicellular, high-order eukaryotic organisms. Despite some of the practical difficulties that are inherently attached to the use of such complex organisms, it has been applied in the context of complex behavior, like memory (7), mating (8), or organismal development (9). It is microbial research, however, in which experimental evolution has become a popular and widely used tool over the last decades. Microorganisms such as yeast, bacteria, and viruses provide major advantages for setting up evolution experiments.

First of all, microorganisms are highly practical, especially with respect to conducting evolution experiments. Since they are small, divide rapidly, and often require only simple growing conditions, large and highly replicated populations can be propagated easily and cheaply to reach many generations on short time scales. A telling comparison can be made between the longest evolution experiment with microorganisms, started in 1988 and still running today (see http://myxo.css.msu.edu/index.html, the experiment's webpage, and see "A special case: Richard Lenski's long-term evolution experiment," below), and a comparable experiment on mice that started only 5 years later, which is presently also still ongoing (10). The former experiment generated over 62,000 generations, while the latter has reached only ±80 generations so far (10). Even extremely long ecological selection experiments on maize and grass that have been running for over 100 years now still add up to only ±100 generations (11, 12). In addition, microorganisms' small genomes and readily available genetic and molecular tools make identifying the underlying causes of evolution much more feasible, which can lead to the unraveling of evolutionary dynamics of adaptation in great detail. The ability to preserve population samples indefinitely from intermediate time points in ultra-low-temperature freezers allows for the construction of a frozen time vault from which evolution can be resumed as a backup for unintentional events. More importantly, it allows one to perform replay experiments, restarting evolution at any given point in time (13), or to compete endpoints against any intermediate resurrected samples to directly compare fitness (14). Furthermore, analyses can be repeated or performed as soon as new and more-sensitive techniques become available (15).

Second, the evolution of microorganisms themselves is also highly relevant for many reasons. Microorganisms have a profound impact on our health, as both microbiotal mutualists and disease-causing agents; are widely used in biotechnological applications in industry; and are vital parts of many ecosystems on the planet, where they constitute the most diverse and abundant group of organisms. In addition, experimental evolution with microorganisms can be used as a low-complexity model system to test evolutionary theories. For example, it helps us understand the process of evolution by natural selection, how specialists or generalists emerge, and how diversity can be maintained but also allows one to obtain insight into more-specific evolutionary phenomena, like the origin of innovations, multicellularity, sexual reproduction, and how species emerge. As such, lessons learned from microbial evolution experiments can result in a better understanding of, for example, cancer progression, since cancer evolution follows a clonal pattern similar to the one of most bacteria. Experimental evolution with cancer has been proposed, as cancer-derived cell cultures share many of the traits that make microorganisms ideal for experimental evolution (16–19), and indeed, the first reports in this field have recently emerged (20–22). Apart from studying evolution, experimental evolution using microbes has become a very popular and powerful tool in other fields as well. The search for the underlying genetic and molecular mechanisms of many complex physiological traits can suffer from limitations of traditional methods that are often based on biased mutant libraries that contain only limited numbers or types of mutants. Nature's unbiased solution for the adaptation of the trait of interest under carefully designed conditions has been shown to aid in untangling complex phenotypes. By selecting for very nuanced or specific changes, evolution in the laboratory has, for example, shed light on how viruses become airborne (23) or how attenuated viruses can regain virulence (24) and how bacteria can develop and use clonal heterogeneity to their advantage (25–27). Similarly, experimen-

tal evolution can be used in certain biotechnological applications in industry, for example, to improve strains for their use in production or to find compounds or environments that elicit desirable evolutionary outcomes, e.g., that select against antibiotic resistance development (28–30). On one side, experimental evolution contributes to a better understanding of traits of biotechnological interest and therefore can lead to direct, knowledge-driven manipulations to substantially amend the biotechnological behavior of strains. Additionally, evolution experiments also allow for further unbiased improvements of the general behavior of species under application-specific conditions. In this case, a full understanding of underlying principles that is often required for enhancing complex traits like stress tolerance or metabolic fluxes for the production of economically valuable compounds is not needed (31, 32).

In this review article, we illustrate the experimental design of evolution experiments, explain what affects the dynamics of phenotype and genotype observed during these experiments, and show how experimental evolution can be applied by the research community in testing ecoevolutionary theories, studying complex phenotypes, and improving biotechnologically important traits. We start by giving an overview of the setups that have been used for experimental evolution using microorganisms such as yeast, bacteria, and viruses. To capture the recent explosion of the field, the examples in this overview are further supplemented by an online database, the "Compendium of Adaptive Microbial Evolution Experiments in the Lab" (CAMEL), which compiles and details studies employing evolution experiments and allows community-driven updates (www.cameldatabase.com/) (see Table S1 in the supplemental material). Next, we broadly discuss the population dynamics and variety in genomes and phenotypes observed during microbial evolution experiments and how the process of evolution is shaped by aspects such as epistasis, the topology of the fitness landscape, second-order selection, and (a)sexual reproduction modes. The spatiotemporal nature of the environment in which adaptation takes place most profoundly affects the dynamics and outcomes of evolution. In changing environments, trade-off costs of specialists compete with the cost of generalism and help to explain why adaptive evolution does not necessarily lead to specialized life forms only. Heterogeneous, structured environments, on the other hand, often contain many different niches and therefore allow diversity to emerge and endure, further aided by factors such as clonal interference (CI) and negative frequency-dependent selection (NFDS). Terms and abbreviations that are used throughout the text can be found in Table 1. In the future, we believe that experimental evolution using microorganisms will further expand and become a widely applied and mature research tool that complements various experiments in many fields, especially combined with ever-improving sequencing techniques and analyses. This work therefore aims to be an introduction to novice researchers, a guideline to those planning to embark upon experimental evolution, and a reference to veterans in the field.

DESIGNS AND PARAMETERS OF EXPERIMENTAL EVOLUTION

General Conditions of Evolution Experiments

Many microbial evolution experiments employ a constant, simple environment that imposes a seemingly straightforward and moderate selection pressure on the organism, which is often the limiting presence of a single essential nutrient (33) like carbon, nitrogen (34–37), phosphorus (38–40), or sulfur (38, 41). Keeping all other parameters (temperature, aeration, culture volume, and other nutrients, etc.) as constant as possible and without strong limitations for bacterial growth, adaptation is confined to a sole limiting resource. In general, parallel populations or lines are propagated at the same time under the same selective conditions. While founded by a common ancestor, separate ancestral clones are preferentially used when starting these parallel lines to avoid a potential skew toward mutations that might already be present initially by chance in the founding clone. These ancestral clones are often also genetically labeled, expressing either a fluorescent protein (42), antibiotic resistance (43, 44), or a specific pattern of resistance to phages or displaying a specific colony color (45). The label allows the detection of external contamination or cross-contamination between lines in

TABLE 1 Glossary

Term	Explanation
Antagonistic pleiotropy	The phenomenon of a gene that controls multiple traits, some of which are beneficial in one environment but detrimental in another
Arms race dynamics	Used in evolution to denote the situation where competing species or coevolving gene sets are shown to be adapting against each other; refers to the arms race of two competing countries, where each country will produce more arms, etc., to outrival the other
Barcode sequencing	Technology to determine the relative frequency of barcoded individuals in a population by sequencing; a random sequence barcode is added to each individual before the evolution expt; intermittently, samples of the entire population are taken, and the region where the barcode is located is sequenced; the relative abundance of barcodes in the sequence data translates directly to the relative frequency of each individual in the population
Bed-hedging	Long-term survival strategy where an individual in a population shows decreased fitness under current conditions in exchange for increased fitness under future conditions that might endanger the population
Bottleneck	A population bottleneck is referred to as a drastic decrease in population size; in exptl microbial evolution, this occurs when a small proportion of a population is used to inoculate the next generations; the size of the bottleneck is an important determinant of the outcome of the evolution expt; mutation accumulation experiments, for example, use the greatest possible bottleneck of only 1 transferred cell per population
Black Queen hypothesis	Situation where selection leads to the loss of a costly but essential function in part of the population; because this function is costly, part of the population benefits from the loss, but the remainder of the population is stuck with the function and cannot get rid of it, because it is essential for the entire population; much like the Black Queen playing card in the game Hearts, the costly function is a burden for those individuals who have to carry it out
Chemostat	Closed culturing vessel that operates by continuously adding fresh medium and continuously removing used medium, including microorganisms, at a constant rate; the vol in a chemostat remains constant, and by adjusting the flow rate of nutrients, the growth rate of the microorganisms can be controlled
Clonal interference	Occurs in a population when 2 or more beneficial mutations arise independently in different clones and compete with each other
Coevolution	Situation where one species affects the evolution of another species that is present
Distribution of fitness effects of random mutations	Gives the relative abundance of mutations with beneficial, neutral, or deleterious effects; it is mainly inferred from mutagenesis or mutation accumulation experiments; this distribution aids in predicting the evolutionary dynamics and the outcome of evolution experiments
Drift	Genetic drift is the process that changes the frequency of an allele in a population due to random sampling; drift is prevalent when populations go through a bottleneck, during which a sample of the original population will be used as a start for successive generations
Epistasis	Phenomenon where the effect of one gene is influenced by interactions with other genes; various types of epistatic interactions exist depending on the resulting phenotype; overall, epistasis is widespread and largely influences the evolution of several phenotypes
Evolvability	Capacity of an individual or population to evolve; it denotes the ability to generate genetic diversity necessary for adaptation through natural selection
Fitness	Quantitative representation of an allele's or a genotype's reproductive success in a given environment
Fitness landscape	Frequently used in evolutionary biology to visualize the relationship between an individual's genotype and the corresponding fitness or reproductive success; it is a 3D representation where the *xy* plane corresponds to the genotype and the *z* axis shows the fitness for each genotype; a fitness landscape can be rough, with multiple genotypes that confer a fitness benefit, or smooth, with only one clear peak that corresponds to a narrow set of genotypes that are beneficial; the space between peaks in the landscape is called a fitness valley and represents genotypes that are deleterious or neutral in a given environment; the fitness landscape is different under every condition
Fixation	An allele is fixed if the frequency of that allele increases to 100% and remains present in all individuals of the population; beneficial mutations can be fixed by direct selection, and neutral or deleterious mutations can be fixed by second-order selection or genetic drift
Fluctuating selection dynamics	Occur when selection on a given genotype fluctuates over relatively short periods of time; this kind of dynamics can occur when the environment changes, rapidly favoring other genotypes over the initially favored genotype
Generalist	An individual that is able to thrive under a wide range of environmental conditions, in contrast to a specialist
Hitchhiking	Process where the frequency of a neutral or deleterious mutation in the population increases due to natural selection acting on a linked beneficial mutation
Indel	Used to denote both insertions and deletions; indels are structural changes in an organism's DNA that usually lead to frameshifts and, hence, to a loss of the gene's proper function

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TABLE 1 (Continued)

Term	Explanation
E. coli long-term evolution expt (LTEE)	Initiated by Richard Lenski, it is the longest-running exptl evolution expt to date; initiated on 24 February 1988, the adaptation of 12 identical populations of *E. coli* to DM25 minimal medium is tracked phenotypically and genetically; the expt is still running and has reached almost 70,000 generations; results from this expt have yielded invaluable insights into evolutionary processes and dynamics, and it still continues to reveal hidden information crucial to completely understand the phenomenon of evolution
Mutation accumulation	In a typical mutation accumulation expt, all mutations, including neutral and deleterious mutations, are allowed to be fixed in the population due to single-cell bottlenecks; these experiments are used to study evolution and genetic variation such as DFE
Morphotype	A type of individual within the same population; in a population, different morphotypes can occur and coexist due to various polymorphisms that result in different types of individuals
Mutator	An individual with defects in DNA replication and repair mechanisms that result in an increased genomic mutation rate
NFDS	In the case of frequency-dependent selection, the fitness of a genotype depends on its frequency in a population; in negative frequency-dependent selection of a genotype, the fitness of that genotype decreases when the frequency increases; it is used mostly in the case of interactions between species; a clear example of NFDS is apostatic selection, where a prey that differs from the rest of the population through a (rare) mutation (e.g., that changes its color) has a higher chance of being ignored by the predator and, hence, has a higher chance of surviving
Next-generation sequencing	Collective name for relatively recent DNA sequencing technologies, such as Illumina, 454, SOLiD, PacBio, and nanopore sequencing, etc.; these technologies allow fast, easy, and cheap massive parallel sequencing of billions of sequences at once
Nontransitive fitness	Case where the fitness of an endpoint does not match the sum of the fitness of an intermediate point and the fitness of the endpoint relative to that intermediate point; this occurs when fitness does not increase steadily during evolution but also periodically decreases
Parallel evolution	Occurs when independent organisms evolve under similar conditions to similar phenotypes whether or not via the same adaptive path; often used interchangeably with convergent evolution
Red Queen hypothesis	Refers to the hypothesis made by the Red Queen in the novel *Through the Looking-Glass* (1871) by Lewis Carroll, explaining why in Looking-Glass Land everyone needs to run to stay in the same place; in much the same way, predator-prey systems often lead to ARD, where constant adaptation is necessary not to dominate but to survive in the presence of the ever-evolving competitor
Selective sweep	The frequency of a beneficial allele will increase in the population due to natural selection; associated alleles near them in the chromosome will hitchhike and also show increased frequencies; this process of increased frequencies of a beneficial allele and an associated allele is called a selective sweep
Stress-induced mutagenesis	Relates to the increased occurrence of mutations in the presence of stress; upregulation of various stress responses results in the activation of error-prone polymerases that erroneously repair damage in the DNA, leading to mutations; the mechanism of induced mutagenesis contradicts the classical evolutionary theory that mutations arise spontaneously
Single-nucleotide polymorphism	A single-base change in the genomic DNA sequence of an organism
Specialist	An individual that is specialized to only one specific environment; it will thrive in that environment but will suffer in other environments, in contrast to a generalist
Standing variation	The genetic variation that is present in a heterogeneous population with more than one allele at a given locus
Trade-off	In evolutionary biology, the situation where acquiring a certain beneficial trait under one condition through genetic changes inherently leads to a cost under other conditions
Transposon	Genetic element that can change position within the genome; usually, the position where it "lands" is random, leading to a disruption of a gene's function
Turbidostat	A specific type of chemostat with feedback between the turbidity in the vessel and the flow rate of the nutrients; in this way, a turbidostat enables the maintenance of a constant population density in the vessel
Visualization of evolution in real time	Relates to the expt of Baym et al. (488), where they used a MEGA plate setup to visualize the evolution of antibiotic resistance in real time
Whole-genome sequencing	Process of determining the DNA sequence of the entire genome of an organism; in the past decade, advancements in NGS technologies have enabled relatively cheap and highly parallel WGS on microorganisms

cases where founding ancestors carried a neutral but differential tag. Another precaution that is often taken is intermediate sampling and storage of the evolving lines, as a backup in case of accidents (46) or as a frozen historical library.

"Time" in Experimental Evolution

Given microbes' large population sizes and high division rates, observable evolution

usually takes place rapidly, and therefore, experimental evolution experiments have been carried out over relatively short time scales. "Time" is generally expressed in number of generations, the average number of divisions for each cell in the population. The cumulative total number of cell divisions has been proposed as an alternative, more-meaningful time unit, since mutations are generally considered to take place during cell divisions (47). Moreover, this unit elegantly scales with population size, an important factor that determines the strength of selection and, thus, of adaptive evolution. The cumulative number of generations is nevertheless seldom used, and more often, the total duration in hours, days, weeks, or years is cited along with the number of generations. The absolute time can be most informative, especially in cases of adaptation during (stress conditions causing) prolonged slow or no growth, during which DNA damage and error-prone repair can result in the emergence of mutants (48) (see "Diversified Use of Standard Setups to a Fully Matured Field of Experimental Evolution," below).

Serial Transfer

In standard setups, evolving populations need to be diluted regularly. In this way, the necessary physical space is created, fresh nutrients are supplied, and superfluous end products are removed, allowing for additional cell divisions, competition between mutants, and, thus, evolution by natural selection. To this end, two main operational *modi* are at hand (Fig. 1). The serial transfer modus groups together all evolution experiments that repeatedly grow populations in batch cultures. Often proceeding through all steps of batch growth with a lag phase and exponential growth and up to stationary conditions, these batches are interspersed with diluting transfers to fresh medium, leading to an average of $\log_2$(1:dilution ratio) generations per cycle. Serial transfer is most popular due to its ease and simplicity and potentially also because of the textbook example of experimental evolution, the long-term evolution experiment (LTEE), which has been running since 1988 (see "A special case: Richard Lenski's long-term evolution experiment," below). As a result, it can be performed in almost any laboratory and scales easily to allow many replicate lines to evolve simultaneously. As many as 748 lines have been maintained simultaneously for 400 generations or 104 days using microtiter plates (49). Maintaining many replicate lines for a long time, as a consequence, is often experienced as being labor-intensive, and human limitations and errors quickly become prevalent. To this end, some groups have automated (part of) the process of serial transfer to maintain some 4,000 populations simultaneously for 500 to 1,000 generations (50–53).

A common criticism on serial transfer is that the conditions are never entirely constant. For example, for every cycle, the population alternates between low and high cell densities, as determined by the dilution ratio. As a consequence, the population also experiences a bottleneck, a source of drift and stochasticity given the random subset of the population that is passed to the next cycle. In this context, the effective population size is often computed as N_e, the size of an ideal population with a constant size, under perfect homogeneity and evolving neutrally with random sampling of alleles from predecessors into the offspring, in which genetic drift or randomness in evolution acts at the same rate as in the actual population (54–56). As such, N_e is a measure of the strength of natural selection or neutral drift present in a population, with a high N_e value being in favor of natural selection and a low N_e value pointing to stronger neutral drift. Actual populations, even in microbial evolution experiments, are often far from ideal, as they proceed through population bottlenecks and suffer from hitchhiking through linked mutations or other effects of population structure. The effective population size is therefore often orders of magnitude lower than the maximum population size and can be estimated in different ways (54). For the serial transfer regime, the approximation $N_o \times g$ is often used, where N_o is the size of the population bottleneck applied at transfer and g is the number of generations during one batch growth cycle (45). In its most extreme form, serial transfers of single-cell bottlenecks are applied at each cycle. N_e in so-called mutation accumulation (MA) experiments there-

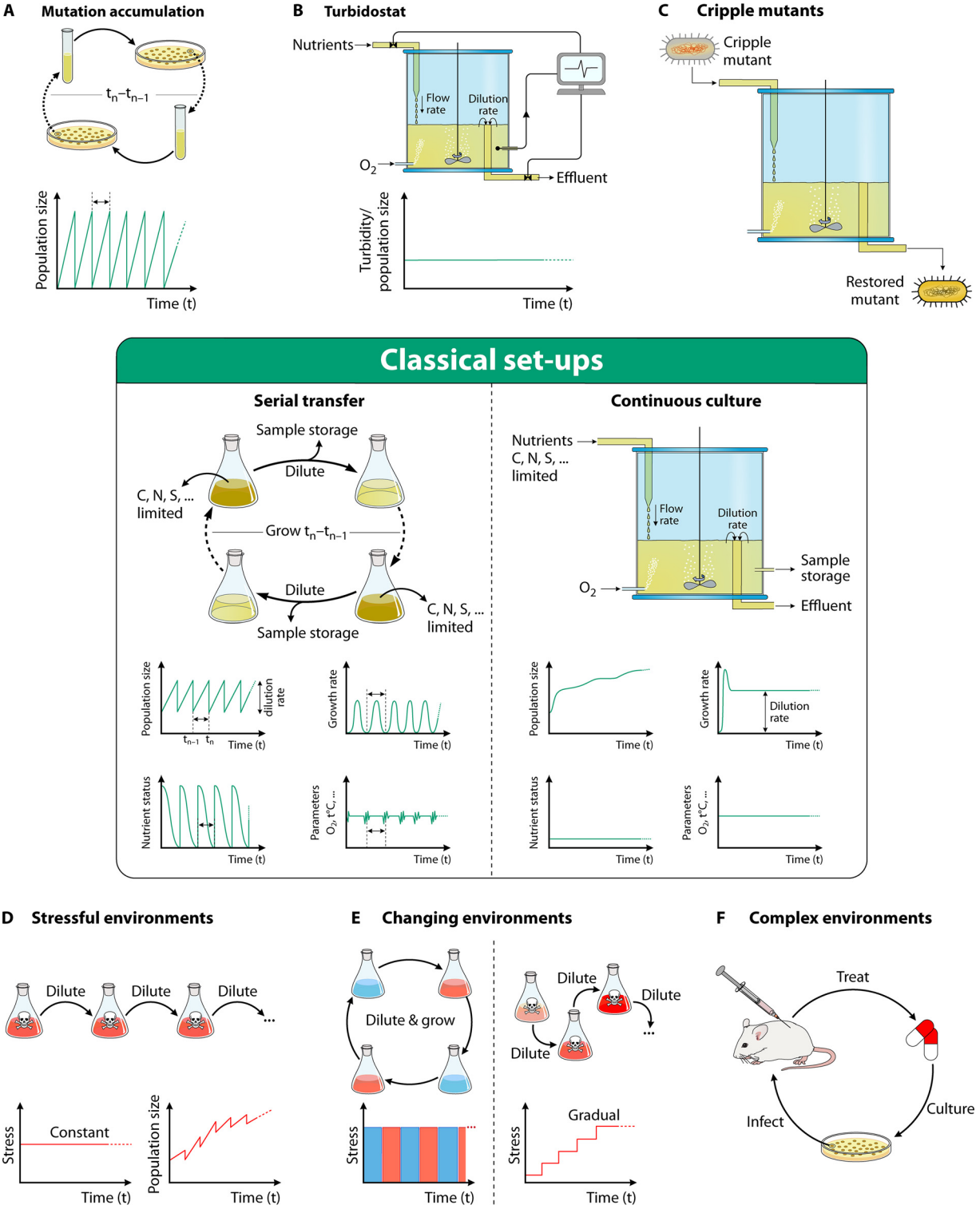

FIG 1 Overview of experimental evolution designs. In the center, the two main classic setups are shown, serial transfer and continuous culture, with illustrative responses of various relevant parameters. Over time, experiments started to deviate from the central designs. For most of the categories shown here, deviation exists based on each of the central designs, but only one is shown as an example. (A) Extreme bottlenecks in serial transfer regimes called mutation accumulation experiments weaken selection in favor of random drift. (B) Continuous cultures no longer operate only at fixed dilution rates but can maintain stable turbidity by a programmed autofeedback loop as in the turbidostat. (C) Starting with crippled mutants, often lacking core metabolic or regulator genes, allows examination of nature's solutions to this internal stress. (D) Additional stress factors are often applied to gradually improve the response of species to these external inhibitors, e.g., by increasing the population size. (E) Adaptation to changing conditions results from either alternating between different selective environments or directionally increasing selective stress during adaptation (with or without a feedback loop). (F) Finally, some evolution experiments try to accurately simulate natural conditions, resulting in complex environments that can differ in time and space, or use multiple (mutant) species simultaneously, e.g., adaptation of microbes in an *in vivo* infection model.

fore approaches 1, and genetic drift dominates evolution, which eliminates the influence of natural selection and allows mutations to accumulate purely by chance (57–61) (Fig. 1A). Along with these alternations between low and high cell densities, the evolving population shifts each cycle from an initially high growth rate to lower ones once the availability of easy-to-use nutrients drops and waste products accumulate (a "feast-and-famine regime" or "seasonal environment" [62]), which results in yet another, often unwanted, change in selective pressure. Thus, the fixed manipulations of serial transfer do not lead to fixed selective pressures, but they rather obscure a continuously variable selective environment that is created. Some studies have tried to mitigate these side effects by imposing more-frequent dilution to keep cells in exponential phase (63–65). Clearly, for long periods of time or when evolving many replica lines at once, this adaptation converts the practical operation of serial transfer into a rather impractical one, except when laboratory automation is available (66, 67). On the other hand, one might argue that many situations in nature resemble regimes of feast and famine or the fluctuation resulting from serial passages, for example, during a pathogen outbreak or in the vertical transmission of symbionts, where each affected host could be regarded as a batch culture of microorganisms.

Continuous Culturing

A second major operational modus to carry out evolution experiments is to culture microorganisms in continuous culturing devices (68). Continuous culturing in a chemostat was introduced in the 1950s independently by Jacques Monod (69) and Novick and Szilard (70), and its use in evolution experiments circumvents several drawbacks linked to serial transfers. Chemostats are closed culturing vessels that operate by continuously adding fresh medium at a fixed dilution rate while simultaneously removing microbial culture at an equal rate (Fig. 1). As such, the volume inside remains constant and is well mixed, usually by aeration (41) and/or stirring (35, 71). Populations grow in a chemostat at steady state with a specific growth rate equal to the dilution rate (72). Thus, the growth rate is precisely controlled by the operator, within biological limits to avoid culture washout, thus with a dilution rate lower than the maximum specific growth rate. Therefore, evolutionary adaptation under different growth rates can be easily monitored using chemostats operating at different dilution rates. Here, the number of generations equals ln(2)/dilution rate $\times$ time (73). The density of the population depends solely on the concentration of a single limiting nutrient in fresh medium. In addition, the dependencies of growth parameters on operational settings are described by differential equations allowing for straightforward mathematical description (69, 74). The operational parameters are most often chosen such that the culture environment mimics the phase in batch growth just before complete nutrient exhaustion. Therefore, the populations are called "poor, not starving" or "hungry" (68).

While chemostats fix the dilution rate and, thus, the microbial growth rate, likely eliciting an increasing population density during adaptation, a turbidostat is set to keep the cell density constant (Fig. 1B). To achieve this, the concentration of biomass is continuously monitored, and the rate of dilution with fresh medium is automatically adjusted in a feedback-like fashion to maintain a desired value (75, 76). A turbidostat often operates with a dilution rate near the maximal growth rate of the cells and with nutrient-abundant environments. While similar to the serial transfer regime, with frequent dilution to avoid changing environments, a turbidostat is clearly superior in precisely maintaining mid-log-phase conditions and selecting for mutants with an increased maximal growth rate. As a result, a continuous culture device should enable the infinite and automated propagation of populations in a truly constant environment without bottlenecks or feast-and-famine regimes.

Despite their theoretical advantages, these systems are used less often than serial transfer in experimental evolution. One reason for this might be the limited relevance of continuous systems to natural conditions, yet microbial evolution in the rumen or in a water treatment plant potentially is best described as a continuous culture. In addition, setting up a chemostat is complex and can be challenging, as is avoiding

TABLE 2 Continuous culture devices used in evolution experiments for which operational instructions are available

Device	Description	Reference(s)
Sixfors	Instructions on how to operate the commercial system of Infors HT/AG and apply it for exptl evolution are provided online	71
The People's Chemostat	Home-built chemostat first built by Bruce Levin in 1973; all the instructions on material, assembly, and how to operate it can be found online	508, 509
Chemostat for applying stressors	Turbidostat-like operation of a chemostat whereby the level of a stressor is incrementally increased by visually monitoring the density of the culture; video can be found online	510
Multiplexed chemostat arrays	Arrays of small chemostats, or ministats, that can be operated in high parallel; the online manual to build these ministat devices is extensive and allows implementation in many laboratories for exptl evolution	41, 511
Versatile continuous culture device	Small-vol, low-cost continuous culture device that can switch easily from chemostat to turbidostat modes and can additionally monitor pH as an indirect metabolic indicator; comes with extensive software support for operational regulation	512
Morbidostat	Device specifically designed to continuously culture microorganisms under dynamically sustained inhibitors; specifically, it was used for studying evolution toward antibiotic resistance; can additionally be used as a chemostat or turbidostat	85, 513
Flexostat/Fluorostat	Miniaturized turbidostat that can be multiplexed to 8 vessels while maintaining its investment costs below $2,000 through the use of 3D-printed material and standard laboratory material or university services; an additional light source allows fluorescence readouts	514
Milliliter-scale chemostat array	Without expensive feedback systems, flow rates can be controlled for 8 chambers independently for doubling times ranging from 3–13 h	515

contamination once the system is running. Therefore, although the system theoretically should run completely automated, regular observations and maintenance are needed (74, 77). Commercial systems are also often costly, which further impedes upscaling and, thus, the propagation of many replicate lines. Generally, only a few replicas have been used, run either in true parallel (35, 78) or sequentially (79). In extreme cases, evolution experiments were replicated 24 times (38) or carried out with no replicate lines at all (39, 80, 81). In addition to their complexity in construction, continuous cultures lack the flexibility for practical adaptations that is presently often desired by many studies (see below) (68). Finally, the application of continuous culture devices in experimental evolution setups suffers from cells that improve their capacity to adhere to the vessel wall, e.g., by improved biofilm formation. Such an improvement will always be beneficial regardless of which unambiguous selective pressure is applied by controlling the operating conditions since these cells never leave the device. Indeed, better vessel wall adhesion or faster sedimentation to the bottom (in- and outlets are usually at the top [82]) not only has been shown to be an evolutionary side effect of evolution in continuous culture devices but also can cause unintended intermediate stops (46) and impede the longevity of the evolution experiment (72, 74, 83).

Lately, many laboratories have provided step-by-step (video) instructions on how to assemble and maintain or build miniaturized and multiplexed continuous culture systems (Table 2). These efforts break down barriers for other researchers to build similar setups and reduce the required actions to basically buying a pump. Further miniaturization to reach a high throughput of over 1,000 parallel populations has become possible but for now remains out of reach for long-term evolution experiments (84). Others have shown that continuous culturing is also feasible for more-complex environments. Variants of a turbidostat, for example, control the culture density not only by dilution but also through the application of growth-limiting stress, like ethanol or temperature, to constrain the growth rate and select for improved growth under these conditions (79, 81, 83, 85) (see "Diversified Use of Standard Setups to a Fully Matured Field of Experimental Evolution," below). Despite all these efforts, continuous culturing remains in general poorly suited to exploring evolution in dynamic environments (68). Side effects of vessel wall adhesion or faster sedimentation have been improved by increasing mixing or using surfactants (41). More-complex systems have

also been developed. For example, in a twin-chemostat system, a second chemostat vessel allows further propagation and evolution of the culture once the first vessel requires cleaning (33). Switching between culture vessels at given time points permits cleaning, turning improved adhesion to the culture vessel wall into a futile adaptation and allowing experimental evolution to be carried out over longer periods, e.g., over 880 days or 10,000 generations (33), an order so far achieved only by serial transfer. Alternatively, wall growth has been avoided by transforming a chemostat into a long transparent tube filled with medium, where a part of the tube is the actual growth chamber, that moves stepwise through the complete length of the tube (86). All these optimizations and adaptations might increase the use of continuous cultures in experimental evolution in the future (68, 74, 87).

Diversified Use of Standard Setups to a Fully Matured Field of Experimental Evolution

To answer more-complex evolutionary questions, investigate complex physiological traits, improve biotechnological properties of species, or better mimic more-natural conditions, numerous evolution experiments that deviate from the classical setups have been devised. Nowadays, these diversified classical setups for experimental evolution have become the most popular implementations of experimental evolution, as it has been shown that they can be fruitfully applied in many distinct topics of microbial research.

Cripple mutants. Cripple mutants, often lacking major regulatory or metabolic genes (49) or carrying malfunctioning essential genes (88), have been used as founders to investigate alternative evolutionary solutions of biology (Fig. 1C). For example, evolution experiments have been initiated with mutants lacking key metabolic enzymes (89–92) or global regulators (93, 94), with mutants having alterations in their central metabolism (95–97), or with mutants lacking genes that were previously shown to be targets of evolution by accumulating gain-of-function mutations, thereby opening the road to alternative paths (98, 99).

Beyond simple nutritional stress. Experimental evolution has also been carried out under more-challenging environmental conditions (Fig. 1D), for example, under extreme pH (100–103), osmotic pressure (101, 104–106), suboptimal redox status (101), oxygenation (107), extreme temperature (67, 108–110), or UV radiation (111); in the presence of antibiotics (50, 112–117), antimicrobial peptides (118–120), and alcoholic solvents (101, 121); and even under microgravity (122, 123).

Similarly to the above-mentioned abiotic stresses, biotic stress has also been imposed on microorganisms during evolution experiments. Experiments allowing predation by protists or infections with phages or, when the study focuses on predators, using new or scarce hosts (124–127) are often performed. This kind of biotic pressure seems to constrain the simultaneous adaptation to abiotic conditions (128).

Spatiotemporally changing environments. In experimental evolution, the imposed environment is not always kept constant but instead often changes over time (Fig. 1E). First, there are evolution experiments where the environment is changed progressively. By changing the environment according to evolutionary progress, researchers have tried to prevent the selection pressure from dropping over time and therefore stimulate further adaptation (129), for example, by increasing antibiotic concentrations (83, 85, 130–132) or the concentrations of other antibacterial substances such as silver nanoparticles (133) and other metals (134), the ionizing irradiation dosage (135, 136), solvent concentrations (32, 65, 137–139), hydrostatic pressure (140), or temperature (79, 141, 142) according to the emerged resistance. In a sense, these experiments were all inspired by one of the first evolution experiments ever performed: William Dallinger enabled protozoa to grow at extreme temperatures in the 19th century by gradually increasing the temperature as soon as the protozoa adapted (6).

Others used fixed rates of environmental change without evolutionary feedback, often resulting in deteriorating conditions. These experiments often aim at understanding whether species can adapt by evolutionary rescue or will go extinct in the context

of global environmental changes. Generally, this is correlated with the rate of environmental change and shows that gradual changes result in greater evolutionary adaptation rather than abrupt ones (143–145). As means to study global change, several kinds of increasing stresses have been used, such as increasing antibiotic concentrations (143, 146), osmotic stress (51, 52, 147), phosphate limitation (148), or hydrostatic pressure and temperature (149–151) or, in the case of virus adaptation, changing to a novel host type (145).

Alternating between two or more contrasting environments, either in cycles (107) or randomly (100, 152) and with various frequencies (153, 154), is another category of environmental change that has been used in experimental evolution (Fig. 1E). Classical examples are shifts between carbon sources (155–157), but others have been examined as well, such as alternations between acidic and basic pH (158), different temperatures (159, 160), light and dark regimes in photosynthetic organisms (161–163), different antibiotics (130) or antibiotic treatment and recovery (26, 164), alterations between hosts and predators (126, 152, 165–167), or freeze-thaw growth cycles (168).

In some experiments, the kind and rate of environmental change are determined by the outcome of evolution itself, i.e., when two or more coevolving species interact with each other and evolve in response to each other (169, 170). The best-known examples are predator-prey systems. For example, coevolving bacteriophages and bacteria often lead to an evolutionary arms race where constant adaptation is needed not to dominate but merely to survive with respect to an ever-evolving counterpart, known as the Red Queen hypothesis (RQH) (originating from the statement that the Red Queen made to Alice in Lewis Carroll's *Through the Looking-Glass* [516], the sequel to *Alice's Adventures in Wonderland* [517], explaining why in Looking-Glass Land everyone needs to run to stay in the same place) (171–175). Also, for commensal and mutual interacting partners, reciprocal coevolution dynamics have been reported (176–179).

In a last group, the selective environment is changed not over time but rather in space, or it simultaneously contains contrasting niches. The combination of different niches, often with various grades of interconnectivity and, thus, migration, leads to a heterogeneous and/or structured environment. The use of two or more carbon sources at the same time in a well-mixed environment is an extremely simple example of such a heterogeneous environment without physical boundaries to migration (155, 156, 180–182). Experimental evolution has also been carried out under more-heterogeneous conditions and in structured environments with less mixing (43, 183–185), like biofilms (176, 186, 187), or in a patchy environment with different antibiotic concentrations (188) or differences in illumination (162).

Evolution under conditions closer to those of natural environments. In an attempt to study adaptive processes that could also take place in nature, conditions in experimental evolution have mimicked natural conditions as close as possible (Fig. 1F). While the environment is still often well defined, it combines (multiple) abiotic and/or biotic stresses, changing over time and/or in space (134, 189). Note that some of the studies cited above also combined several stresses although they did so in a generally less extreme fashion and often were not focused on the effect of complex selective forces on evolution. As an example, *Pseudomonas fluorescens* was evolved in a structured environment with a combination of protists, phages, and antibiotics (190), or the adaptation of *Pseudomonas aeruginosa* was monitored in artificial sputum medium to mimic the lungs of cystic fibrosis patients (113, 191, 192). In a less-defined setup, a *Lactococcus lactis* plant isolate was domesticated to a dairy niche by adaptation to milk, which resulted in properties highly similar to those of *L. lactis* isolates from dairy products (193). A similar domestication took place in *Burkholderia cenocepacia* evolved on onion extracts, which resulted in the loss of its pathogenicity to the nematode *Caenorhabditis elegans* (194).

Experimental microbial evolution has also been conducted *in situ*, for example, in eukaryotic cell lines (195, 196), and using whole-animal or plant model hosts such as mice (197–200), corn (201), *Mimosa pudica* (202), rabbits (203), ferrets (23), worms (204), or caterpillars (205), often with the goal of understanding pathosymbiotic adaptation or

how hosts can affect the evolution of the microorganism. These kinds of experiments can serve as nice parallels to observations made based on comparing isolates from long-term infections or symbioses in real life (56, 206, 207). In an analogous way, active efforts are being made, especially by the groups of Buckling and Brockhurst, to monitor the evolution of focal species in communities and/or living in structured and complex microcosms, better resembling natural, free-living conditions. Many current experiments still use only a rather simple binary setup of two species (208–211) that often fall within the coevolving regime of predator-prey systems or beneficial interactions between two species (see also above). However, the use of more-complex communities is emerging. Here, one attempts to understand how ecological interactions can affect the evolution of the focal organisms or how evolution can affect ecosystem functioning. Local adaptation of focal species could, for example, slow down due to strong interstrain competition leading to strong population bottlenecks. Alternatively, evolution might speed up due to fast coevolution between strongly interacting species or take other directions altogether. Depending on the specific system under study and the strength and sign of the ecological interactions present in the community, both outcomes have been observed. For example, adaptation of *P. fluorescens* is constrained when strong competitors are present (212–214), while local adaptation of the species was shown to be potentially equally as important to community structure as the presence of the species itself (215). In contrast, evolution elicited stronger changes when 5 decomposer bacteria, all isolated from the roots of beech trees, were propagated together in a community than those elicited by evolution in monoculture. Not only did stronger metabolic interactions emerge in the form of diverged resource use and waste product cross-feeding, but communities were also more productive (216). Performing evolution experiments with communities can also lead to fairly unexpected and somewhat counterintuitive results. Interspecies gene transfer between *Pseudomonas putida* and *P. fluorescens*, for example, was recently shown to be inhibited in soil microcosms when positive selection was applied for traits encoded by the conjugative agent, a mercury resistance plasmid (217).

All these conditions together in which experimental evolution has been performed listed in this section show its power as a research tool and explain how the field exploded and diversified in many complex and specialized subdomains (Fig. 1F).

A special case: Richard Lenski's long-term evolution experiment. On 24 February 1988, Richard Lenski started culturing his famous 12 parallel *Escherichia coli* populations (see http://myxo.css.msu.edu/ecoli). He used a constant and simple environment. The cultures were grown in minimal medium with low levels of glucose as the sole accessible carbon source, and 1% of each population was transferred daily into new flasks with fresh medium, allowing for another cycle of overnight batch growth (with shaking at 37°C and at 120 rpm). His evolution experiment is therefore an example of a standard serial transfer setup. Being very well thought through, it contributed to current unwritten "laws" to be followed when starting evolution experiments. For example, as a common ancestor, he used two isogenic variants of an *E. coli* B strain that had a rare combination of sensitivity to phage T5 (confirming that it is *E. coli*) and resistance to T6 (most *E. coli* strains are sensitive) (45). Moreover, REL606 and REL607 (six populations founded by each) differed by a neutral, visual Ara marker. Therefore, not only can contamination from external sources be easily detected, but cross-contamination between the separate populations can also regularly be checked for since the handling of the cultures was always performed by alternating Ara^+ and Ara^- populations. Afterwards, this same marker was also used for determining fitness in head-to-head competition experiments. Along with the additional precautions taken (keeping the transferred flasks in the fridge for one night and regularly preserving population samples by freezing), these measures would later add up to the characteristic (and, to outsiders, seemingly excessive) caution taken by anyone working in the field today.

It is not only the clever experimental design of Lenski's experiment that deserves a special mention of his work, as other ingenious evolution experiments with microbes

were also carried out during that time (46, 82, 218–221). The experiment especially deserves an extended discussion because it is still running today, hence its name "long-term evolution experiment" (LTEE). This experiment led to a plethora of results ever since (more than 80 publications on the LTEE alone [see http://myxo.css.msu.edu/ecoli]). Indeed, over the past 30 years and over 68,000 generations (theoretically 6.67 a day, corresponding to more than 1,000,000 years in human terms, while our own species, *Homo sapiens*, is only ±7,500 generations old [222, 223]), the populations evolved and changed (224), resulting in numerous, sometimes unexpected, observations, many of which are used as examples throughout this review. For example, the cell size and growth rate increased (225, 226), while lag times became shorter (62), the cell shape changed from rods to more-spherical cells (227), mutators emerged (228), two or more genotypes coexisted for many generations (229, 230) or interfered with each other in a race to fixation (226, 231), and indirect, sometimes correlated, responses to other, naive environments occurred (232–234). Novelties evolved, such as the capacity to use citrate as a carbon source (13), and elemental stoichiometry changed such that evolved cells contained relatively more phosphorus and nitrogen than carbon, as these elements are abundant in the LTEE environment (235).

The mutations responsible for many of these changes have been identified over the years and are of all kinds, either single-nucleotide polymorphisms (SNPs), insertions-deletions (indels), or larger rearrangements (236–238), but combined, they show a strong signature of natural selection (239). Furthermore, they were often found to interact epistatically in their final, combined result on the phenotype (240–242). Sometimes, several mutations emerged seemingly simultaneously in the same background and were fixed as clades (231). As such, the LTEE combines in one experiment many of the evolutionary observations reported for all other evolution experiments together and consequently enables the testing of evolutionary theories on a longer time scale. Adaptation in the LTEE slowed down over time, but it has not stopped, and according to the power-law model, without any upper boundary that best describes its trajectory, it probably never will (45, 243).

DYNAMICS OF EXPERIMENTAL EVOLUTION

Evolutionary dynamics in experimental evolution largely depend on two aspects: mutations and their effect on the phenotype. To identify mutations in a population, great progress has been made by the development of sequencing technologies. The effect of these mutations, on the other hand, is usually described by the abstract parameter of fitness, which is the actual target of natural selection. In addition, evolutionary dynamics are also influenced by consequences of asexuality and interactions between mutations, also known as epistasis, aspects that help to explain why natural selection does not always lead to the emergence of individuals with the most-optimal set of properties.

Pinpointing Genetic Changes, a Revolution Started by Next-Generation Sequencing

Mutations are the ultimate cause of diversity for selection to act upon and for evolution to take place. Since microbial evolution experiments are commonly started with an isogenic ancestor and no recombination takes place, within this setup, mutations are the only source of variation. Probing the genetic diversity has, for a long time, been far from trivial. Like many fields, experimental evolution greatly benefits from technological advances in diverse areas, but we argue that especially the progress in sequencing technologies has been responsible for the current popularity and frequent use of evolution experiments.

Initial attempts at identifying genetic changes that emerged during the experiment proved difficult. The number of causal mutations was once estimated based on the trajectories of phenotypic traits that often showed sudden changes and thus meant spreading of mutations (45, 82). So-called marker divergence studies made this process easier by clever designs in which mixtures of differently marked ancestral strains are used as the starting culture, also recently renamed as a system for visualization of

evolution in real time (VERT) (244). The deviation of easily detectable marker frequencies from normal fluctuations implies the spread of beneficial mutations (32, 42, 218, 245, 246). These designs are still in use today albeit often serving purposes other than just counting the number of adaptive mutations, since they can also provide information on the selection coefficient of spreading mutations and allow profiling of the distribution of fitness effects (DFE) among all arising mutations in a population (see 'The Overall Phenotype on Which Natural Selection Acts: "Fitness",' below) (247, 248). In some cases, expected target genes were analyzed by direct sequencing based on Sanger sequencing technology, which provided possible causal mutations (143, 172, 228, 249, 250). With small-enough genomes (e.g., viruses), direct sequencing by Sanger sequencing was feasible (126, 167, 189, 251). Others focused on larger changes because they can be easier to observe. Fingerprinting methods allow the detection of mutations involving insertion sequences or transposons (40, 109, 236, 237, 252). Through genetic mapping by conjugative mating (135) or the construction of mutation libraries with subsequent screenings (25, 63, 176, 226), causal genetic changes have also been identified.

All the above-described methods were largely abandoned once next-generation sequencing (NGS) techniques emerged, which enabled convenient whole-genome sequencing (WGS) of microbes. One of the first reports still combined NGS with traditional and laborious shotgun Sanger sequencing (253). Indeed, many of the early uses of NGS to resequence evolved clones still suffered from drawbacks due to the error-prone nature of the early techniques (42, 254, 255). Over the years, technology improved, and mutation identification was performed on many different platforms, such as SOLiD from Applied Biosystems and Life Technologies (114, 131, 256), pyrosequencing by 454 Life Sciences and Roche (23, 157, 171, 257), and different comparative genome hybridization techniques (38, 42, 96, 156, 254), or by using a combination of various platforms (35, 37, 92, 238, 258). Recently, sequencing by synthesis on the Illumina platform has taken the upper hand (see Table S1 in the supplemental material) (85, 110, 128, 130, 132, 188, 241, 259, 260).

Based on these WGS technologies, all possible types of mutations have been identified in adapted clones. Reports on the outcome of evolution experiments are dominated by the importance of SNPs, although larger genomic rearrangements, like insertions, deletions, and inversions, are also detected. These larger changes, often related to the mobility of some genetic material (transposons and temperate phages), have been shown on numerous occasions to accelerate or lead to more-parallel evolution (192) and can lead to very specific promoter capture (98, 241) or gene fusion events (261) necessary for evolution to proceed. Nevertheless, the variety of mutations shows the unbiased nature of evolution by natural selection. In addition to clones, sequencing of whole populations (popSeq) is becoming increasingly convenient. In this way, frequencies of mutations on a genome-wide scale within populations have been estimated (117, 262) over different time points (15, 83, 178, 187, 199, 260) and reliable down to frequencies as low as 1% (263), thereby producing detailed snapshots of genetic diversity throughout evolution. Without NGS techniques, interrogation on a genome-wide scale was impossible except for very small genomes of viruses. To detect mutant frequencies, one had to rely on approaches like Sanger sequencing of many clones or population samples (26, 37, 38, 255, 258) or other PCR-based assays (42, 231, 254). While these pre-NGS techniques are limited to known target regions, for the time being, they may still outcompete NGS due to a lower detection limit, greater ease of use, or lower cost (231, 264, 265).

Apart from further improvements in read length, accuracy, speed, output, and cost (266), future applications of NGS techniques in experimental evolution on species with small genomes will benefit arguably even more from improvements in data analysis, sample preparation, and flexibility in multiplexing (i.e., the combination of barcoded samples in the same sequencing run). Recently, for example, many papers have reported large reductions in cost and time for the preparation of sequencing libraries by using customized workflows (267, 268). At the same time, these techniques deliver

a tremendous increase in multiplexing; up to 4,000 samples have been combined, while theoretically up to 36,864 unique barcodes could be generated by combining any of the 192 forward and reverse 8-base barcodes that passed the applied filtering rules to ensure high quality and maximum demultiplexing (268). In the analysis of popSeq data, recent efforts have allowed the accurate prediction, on a genome-wide scale, of the separate haplotypes that are present in evolving clonal populations (269), information that remained hidden until now because of the short read lengths. Additionally, the information on many time points throughout evolution can be combined to improve the detection limit and the haplotype assembly (270).

The Overall Phenotype on Which Natural Selection Acts: "Fitness"

Whereas identifying genetic changes is nowadays straightforward, demonstrating a link between genotype and phenotype is not. Often, specific lines of evidence can help in identifying the possible causal one(s): intragenic mutations changing amino acid sequences of the encoded protein, or nonsynonymous mutations, are more likely to cause phenotypic changes than intergenic or synonymous mutations, and parallelism between multiple independent lines can further point to causality. However, beneficial synonymous mutations were selected during experimental evolution in *Methylobacterium extorquens* (271) and *P. fluorescens* (272). Furthermore, genetic parallelism can also be mutation driven when mutational bias is strong and the genome is small, as for bacteriophages (273). The strongest argument for an important contribution to the phenotype, though, can be delivered only by some form of genomic engineering where mutations are replaced by the ancestral allele or reconstructed in the ancestral background and a corresponding change in the phenotype is observed.

It is indeed the phenotype, not the genotype, that is the direct target of natural selection, with a mutant's fitness as the ultimate target. This abstract parameter describes the reproductive success of a genotype in a given environment (274). Many relevant growth parameters have been used as a proxy for absolute fitness, such as growth rate (50, 64, 90, 132, 242), yield (81, 131, 160, 190), lag phase (275), or others (26, 62, 276) (see Table S1 in the supplemental material). Fitness actually combines all contributing factors together. Moreover, a mutant's fitness makes real sense only in comparison to a competitor, which is what actually happens during evolution.

Usually, relative fitness is measured in direct head-to-head competition experiments, where mutant and ancestor are mixed and grown under conditions identical to those of the evolution experiment itself (Fig. 2A). Based on the frequencies of mutant and ancestor at the beginning and at the end, the fitness, W, of strain A relative to strain a is then often approximated as the ratio of the number of doublings of each strain or the relative growth speed over a given time interval (243), $W_A = M_A/M_a = \ln(A_f/A_i)/\ln(a_f/a_i)$, where M_A and M_a are the approximated exponential growth rates or Malthusian parameters, A_i and a_i are the initial densities, and A_f and a_f are the final densities of strains A and a.

This formula becomes troublesome, for example, when populations decline during the competition experiment (see http://myxo.css.msu.edu/ecoli). Therefore, other, more-abstract or more-exact formulas also exist (274) and have been used in experimental evolution (38, 277). In each case, however, genotypes with a relative fitness value above 1 are called adaptive or beneficial and generally have an increased frequency, while neutral and deleterious mutations have a fitness value equal to or below 1, and their frequency will decline or be maintained during the course of evolution. In an analogous way and in marker divergence studies, fitness can be estimated along the evolution experiment. In this case, the experiment could actually be considered a long-term competition experiment starting from a library of differentially marked ancestors that will eventually acquire mutations and diverge. Here, the rate of change in marker frequency is an actual measure of relative fitness compared to the average population (Fig. 2B) (247).

The fitness landscape and the distribution of fitness effects. To conceptually visualize the fitness of all possible genotypes in a given environment, Sewall Wright

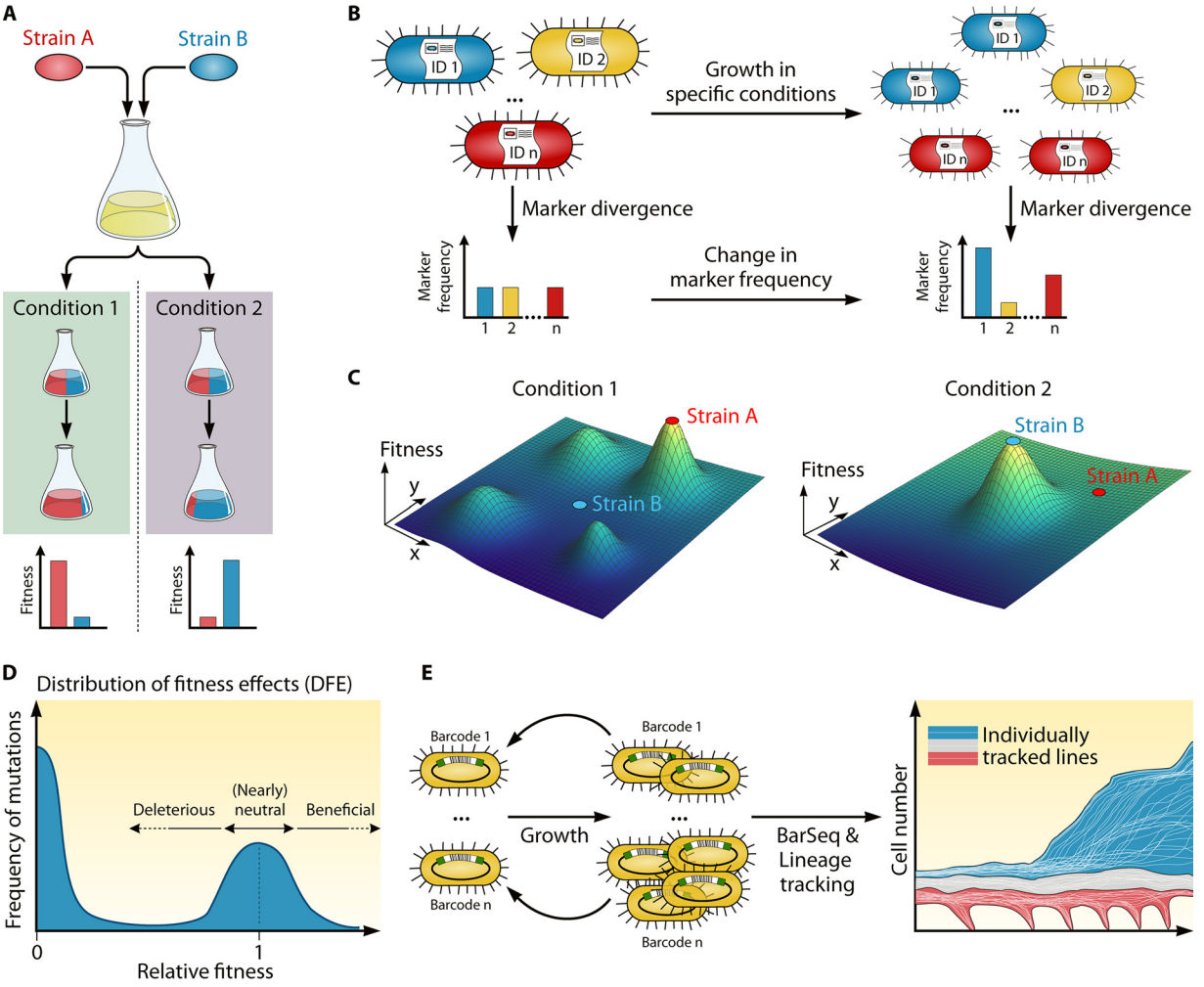

FIG 2 Fitness, fitness landscapes, and distribution of fitness effects in microbial evolution experiments. (A) When two strains are grown together under different conditions, determination of their respective abundances after a defined growth period can be used to measure their fitness under those conditions. (B) By employing markers and starting with a mixture of strains, the fitness of sweeping mutations can be monitored by monitoring the divergence of markers in time. (C) Fitness landscape showing the fitness (*z* axis) for each genotype (*xy* plane). The fitness landscape under condition 1 is rugged, consisting of several fitness peaks. Strain A is located at a fitness peak and outcompetes strain B under condition 1. The fitness landscape under condition 2 is simple, showing only one single fitness peak. Under this condition, strain B is more fit than strain A. (D) The diversity of effects of a mutation can be visualized as a distribution of fitness effects (DFE). Most of the mutations have a deleterious effect and will rapidly disappear from the population. The frequency of mutations with neutral or near-neutral effects follows a clock-like distribution, resulting in only very few highly beneficial mutations. (E) Barcode sequencing (BarSeq) combines random barcoding of individual strains with high-throughput monitoring of the abundance of mutants, which directly translates to the mutant's fitness (506, 507). The BarSeq approach has been successfully applied to track lineages with ultrahigh resolution and high throughput in experimental *Saccharomyces cerevisiae* populations (248). Some sublineages acquire a beneficial mutation and have an increased frequency, and other lineages acquire a deleterious mutation and go extinct. Some lineages acquire a neutral or nearly neutral mutation, resulting in a nearly unchanged frequency. (Panel E based on data from reference 248.)

conceived the metaphor of a fitness landscape (518). Here, a three-dimensional (3D) surface represents all possible genotypes (a simplified version of the actual multidimensional space), peaks denote fitness optima, and valleys represent genotypes with low success (Fig. 2C). Evolution can be seen as a walk on this landscape, usually toward higher fitness peaks under sufficiently strong selection. Representations of such landscapes generally contain few peaks (rugged landscape) or only one peak (smooth landscape), with many vast planes and valleys (Fig. 2C). Although the shape of the landscape points to other features as well, it proposes a plenitude of neutral and deleterious mutations in the genotype space. Microorganisms have encountered both smooth landscapes (240) and rugged ones (255, 278) in experimental evolution. Few landscapes have been reconstructed and often provide only an incomplete image,

mainly because they are small or contain only combinations of mutations that were identified at the very end of an evolution experiment, thereby often ignoring genotypes with an improved fitness that did not make it as well as deleterious or neutral mutations. Still, for small genome sizes at least, genome-wide single-nucleotide fitness landscapes were established, as for poliovirus (279), and more and larger landscapes are being constructed frequently nowadays, aided by extensive sequencing and fitness determinations, for example, resulting in a large fitness landscape in yeast adapting to limiting glucose concentrations (280). MA experiments, in which all spontaneous mutations are allowed to be fixed by single-cell bottlenecks at transfer, except for lethal ones, confirm the abundance of deleterious and neutral spontaneous mutations compared to beneficial ones. Indeed, in these experiments, the fitness of most replicate lines tends to decline over time, which has been dubbed Muller's ratchet, as in these setups, the evolving species has no way to lose deleterious mutations (48, 57, 59, 60, 281). In general, the rates of spontaneous mutation are highest for neutral and then deleterious mutations and lowest for beneficial mutations (14).

Many researchers have tried to profile the distribution of fitness effects (DFE) of spontaneous mutations (58, 61, 282), a key to understanding or predicting biological adaptation (Fig. 2D). While MA experiments are generally skewed toward the abundant deleterious mutations (283, 284), marker divergence studies can be a suitable additional way to pinpoint the DFE of new beneficial mutations (Fig. 2C) (199, 245, 285). An excellent example is a recent report in yeast where the frequency of 500,000 differently DNA-barcoded sublineages of a population was monitored by deep NGS during adaptation under glucose limitation (Fig. 2E) (248). Those authors found 25,000 of them to have acquired a beneficial mutation; most of them had a small effect (1.02 to 1.05), and some carried a larger fitness benefit (peaks at 1.07 to 1.08 and 1.10 to 1.11), but none had any higher fitness. Follow-up work on isolated clones confirmed these findings in the construction of a broad landscape linking fitness to specific single mutations (280). Surely, any aspect of the fitness landscape or DFE depends on the selective conditions, the sensitivity of fitness assays, and the number of mutations examined, which might also explain the different shapes that have been reported for the DFE (286–290). Remarkably, the DFE of beneficial mutations found by deep NGS of the barcoded population does not resemble any of the previously proposed ones (248). In general, however, it seems to be true that the number of beneficial mutations drops once the effects become larger and that while beneficial mutations are rare, mutations with a strong beneficial effect are even rarer (Fig. 2D) (288).

Consequences of Asexuality and the Benefit of Sex

The often asexual reproduction of microorganisms used in experimental evolution has important consequences for the dynamics of evolution, as it does not allow for different genotypes that emerge simultaneously to recombine. Under specific conditions, beneficial mutations can be so rare that their supply rate limits the speed of evolution and that the next mutation arises only after the previous one has swept to fixation (periodic selection or clonal replacement) (291). In this situation, clonal reproduction has few consequences, as the fate of a beneficial mutation will be directly proportional to its own fitness. When a mutation is rare, fitness defines its propensity to survive random effects, i.e., genetic drift. Second, fitness determines the strength of a mutant's selective sweep, i.e., how fast its frequency increases in the population, and thereby also the time for it to reach genetic fixation and replace the previous genetic background for future mutations to emerge in. Consequently, evolutionary dynamics depend only on the waiting time (the beneficial mutation rate and the population size) and the distribution of fitness effects of newly arising mutations (292).

Most evolution experiments operate under conditions with sufficiently high mutation rates in sufficiently large populations for multiple mutations to be present simultaneously (248). The chances that some of these mutations occur in the same background are small, and most mutations likely occur in different individuals. At this point, the reproduction mode of organisms determines whether or not recombination can

occur and has consequences for the evolutionary dynamics. Specifically, if propagation is entirely clonal, purging of deleterious or neutral mutations from a haplotype is difficult, as is the combination of different beneficial mutations in different backgrounds.

Clonal interference. When beneficial mutations emerge in different individuals, an evolving population harbors different mutant sublineages. In the absence of recombination, only one of these mutations can ultimately sweep to fixation, while the remaining ones will be outcompeted along with the ancestor. The resulting competition among mutant clones, in addition to the competition between a clone and its ancestor, is called clonal interference (CI). It has been shown to be prevalent in evolution experiments, observed by either NGS (187, 263, 270, 293) or marker divergence studies (42, 199, 245, 248, 285) or based on phenotypes of separate clones (13, 294), and clearly influences the dynamics of evolution.

Intuitively, the mutant with the largest fitness advantage should eventually become the dominant one. However, the fate of such a genotype becomes uncertain by CI. Indeed, during the sweep to complete fixation, a more beneficial mutation can arise in a different background. In that case, the frequency of the mutation that was initially the most beneficial will increase only to a certain level in the population and then decrease again in favor of the new mutant. As such, CI promotes the fixation of genotypes with large fitness improvements, even if they are rare. Based on empirical data, beneficial mutations with small effects are indeed quickly outcompeted in favor of prior fixation of mutations with large fitness increases (231, 263, 270, 285, 294). In extreme cases, when a genotype that is almost fixed is outcompeted by a mutant that emerged in a background that was hardly detectable because of its very low frequency, this has resulted in the so-called leapfrog phenomenon, as the dominant genotype is replaced by a mutant that is more closely related to the founding ancestor than to the dominant genotype itself (79, 231, 295).

Even when no fitter mutants arise, new additional beneficial mutations will still keep arising in the competing backgrounds, thereby decreasing the benefit of the most-fit sublineage. The fixation sweep will slow down, again allowing more time for mutants with higher fitness to arise (187, 226, 248, 294). Thus, the rate of adaptation cannot be increased indefinitely by increasing the population size or the beneficial mutation rate. From a certain point on, CI will start to act as a speed limit since its strength scales well with the population size or with the beneficial mutation rate. Larger populations or higher beneficial mutation rates might increase the probability of a mutation with a large effect to occur, yet at the same time, more different mutant sublineages will be present and slow down its sweep further (243, 246, 296, 297).

Genetic hitchhiking. At times, multiple mutations also cooccur in the same background. In large populations, mutants with multiple beneficial mutations often arise and spread in the population like a cohort (on the same background) (15, 231, 270, 298). Cohorts can occur when mutations arise and start spreading in a mutant background before this background has reached fixation, so-called nested fixation (270). Sometimes, multiple mutations arise seemingly at the same time in the same background. The latter, although possible, is very unlikely and probably just a side effect of our limited ability to detect single mutants at a very low frequency. Consequently, a substantial part of the time that it takes for the frequency of single mutants to increase remains undetected, and it is during this time that additional mutations can occur (231). Indeed, in the absence of recombination, mutations in a haplotype are completely linked and therefore can use one another as a piggyback ride uphill in the fitness landscape, a process called genetic hitchhiking. The frequent occurrence of sweeping cohorts instead of single mutants also seems to indicate that the combined effect of mutations is needed for an increase in frequency, to overcome drift or CI. Cohorts arising during adaptation of *Saccharomyces cerevisiae* in rich glucose medium, however, were recently shown to mostly contain only a single driver mutation per cohort (299), yet the cases where multiple beneficials drive cohort expansion might have profound effects, and the number of their occurrences and their consequences potentially also

depend on the specific conditions (population size, mutation rates, and selective pressure). In cases where two or more beneficial mutations that generally do not genetically interact are involved (epistasis [see below]), the term "quasihitchhiking" has been used (270), and the mutants are generally called codrivers, as they aid each other in their fixation (231).

Strictly speaking, the term genetic hitchhiking is limited to the situation where neutral or deleterious mutations are being fixed. Natural selection would normally purge single deleterious mutations from the population, while neutral mutations could be lost or are expected to be fixed only very slowly in large populations by drift (292). However, during asexual reproduction and, thus, the complete linkage of the entire genome, neutral mutations have been shown to have an increased frequency and can become fixed in the population by hitchhiking with beneficial ones (38, 175, 255, 270). Hitchhiking of deleterious mutations has been observed less frequently (148, 300). While neutral mutations are often regarded as neutral passengers (15), hitchhiking of deleterious mutations is less likely, as they probably hinder the beneficial effect of the driver. Only under specific conditions where mutations interact with each other to change their effect on fitness can deleterious mutations arise along with a beneficial one, which masks the harmful effect. Interactions of this kind are extensively discussed in the section on epistasis below.

Sexual reproduction and recombination speed up evolutionary adaptation. Sexual reproduction is common among different taxa of organisms in nature. However, the possibility of having sex comes with a substantial cost. Only recently have people started to understand the reasons why sex is so pervasive (301). Over the years, many hypotheses were formulated to explain the emergence and maintenance of sexual reproduction, such as necessity, but generally speaking, all of them can be brought down to the theoretical prediction that the absence of recombination, through both CI and genetic hitchhiking of deleterious or neutral mutations, places a (speed) limit on adaptation (295). In the absence of sex, a beneficial mutation remains linked to a specific genetic background, and hence, the overall effect of that mutation largely depends on the fitness of that background (302). Sexual reproduction could offer advantages here, and CI has therefore been proposed as one possible driver for its maintenance (303). Indeed, sex unlinks mutations from their genetic background, thereby making natural selection on beneficial mutations more efficient and releasing the speed limit on adaptation in sexual populations (the "ruby in the rubbish effect") (304–306). This hypothesis is backed up by data that show a greater strength of selection on new mutations when recombination was present in *Drosophila melanogaster* populations (302). In contrast, earlier data show increased fitness in the case of sexual reproduction in an environment to which the population was well adapted but not in a new environment in which adaptation was necessary. This observation supports the hypothesis that sex aids in purging deleterious mutations rather than increasing the efficiency of selection of beneficial mutations (307). Overall, it is generally accepted that sex increases the rate of adaptation, but the underlying evolutionary dynamics remain unclear.

New technologies have recently offered the possibility of addressing the long-standing question of why sex evolves and persists despite its high cost from a genomics point of view (301). The genetics of 6 sexually evolving yeast populations were compared to those of 12 asexually evolving populations. Both groups accumulated similar proportions of synonymous, nonsynonymous, and intergenic mutations. While all types of mutations were equally likely to be fixed in asexual populations, predominantly nonsynonymous mutations were fixed in the sexual populations. Thus, sex altered the molecular signatures of adaptation, suggesting that sex and recombination indeed improve the efficiency of selection to fix beneficial mutations and purge deleterious ones (301). These results were confirmed in asexual *E. coli* strains that were converted to high-frequency-recombination strains by the genomic incorporation of the F-plasmid conjugation machinery and the removal of surface exclusion factors so that bidirectional DNA exchange can occur (308). Recombination within the "gender-

less" *E. coli* population accelerated adaptation in evolution experiments in comparison to a recombination-deficient wild-type strain by alleviating clonal interference and combining beneficial mutations in one background (308–311). Previously, attempts to increase the rate of adaptation in evolved strains of the LTEE by importing new genetic variation through similar rounds of bacterial sex did not result in increased adaptation (312). In contrast to the work of the group of Kao, the purpose of sex was to import *en masse* variation from distant *E. coli* strains to speed up the process of adaptation. However, as became clear recently, this can overwhelm selection and lead to the fixation of donor alleles without a positive effect on recipient fitness (313). The combination of more variation by starting from standing variation rather than clones and sexual reproduction improved selection in *S. cerevisiae* populations, as it resulted in sustained gradual adaptation over long periods of time by breaking down linkage disequilibrium and transitioning from a selection of genotypes to one of beneficial alleles (314). In asexual populations, in contrast, adaptation was slowed down after the first sweep to fixation that purged all diversity on which natural selection could act.

Epistasis Is Everywhere

So far, we assumed that mutations act independently from each other. The effect of a mutation is then the same whether it arises in the ancestral background or in a background already carrying a mutation. The extra mutation simply adds to the selective effect of the initial mutation or has a multiplicative effect toward its fitness. Although mutations with independent effects have been found in experimental evolution (38, 315), mutations often interact with each other, making their combined effect difficult to predict from their individual effects. Mutational interaction or the conditional fitness of mutations, depending on the genetic background, is widely known as epistasis and is of great importance in the dynamics of evolution. It can result in rugged fitness landscapes or transform the static notion of landscapes and that of the DFE of new mutations into a more dynamic one that changes over time and during evolution. Epistasis can make evolution contingent on history, have an impact on the paths that adaptive walks follow, and cause innovations to arise.

In general, epistatic interactions can be divided in two groups, either negative or positive, depending on whether the combined effect of the mutations results in a lower or higher fitness than expected (Fig. 3A). The numerous examples of positive or negative epistasis, together called magnitude epistasis, highlight its widespread nature and suggest that, certainly with respect to future improvements of fitness measurements to detect the smallest changes, neutral interactions might be truly exceptional (316). Although this subdivision is straightforward with regard to any combination of mutations, many additional, somewhat overlapping but nevertheless useful forms of epistasis have emerged along with increasing observations of epistatic effects (Fig. 3).

Antagonistic or diminishing-returns epistasis. The outcome of a combination of mutations can be less extreme than expected (Fig. 3E). For example, two beneficial mutations can result in a double mutant with lower-than-expected fitness, or two deleterious mutations can have less of a negative effect than calculated based on their separate effects (without resulting in a respectively lower/higher effect than any of the mutations separately). This interaction is known as antagonistic epistasis and is the most abundant form of epistasis observed so far (for specific examples, see references 266, 290, and 307–310).

Apart from specific examples, antagonistic epistasis has been found to be a general theme among beneficial mutations in experimental evolution (240, 255, 259, 317–319). Being so pervasive, it is therefore also held responsible for the generally observed declining rate of fitness evolution during adaptation in a simple environment that is held constant. Under these conditions, the gradual fitness increase is strongest in the beginning but fades over time (reaching a predicted rate limit [243, 320]), bearing a sign of diminishing returns, and therefore, antagonistic epistasis has often been dubbed diminishing-returns epistasis.

Indeed, in a recent large-scale experiment with yeast, populations founded by

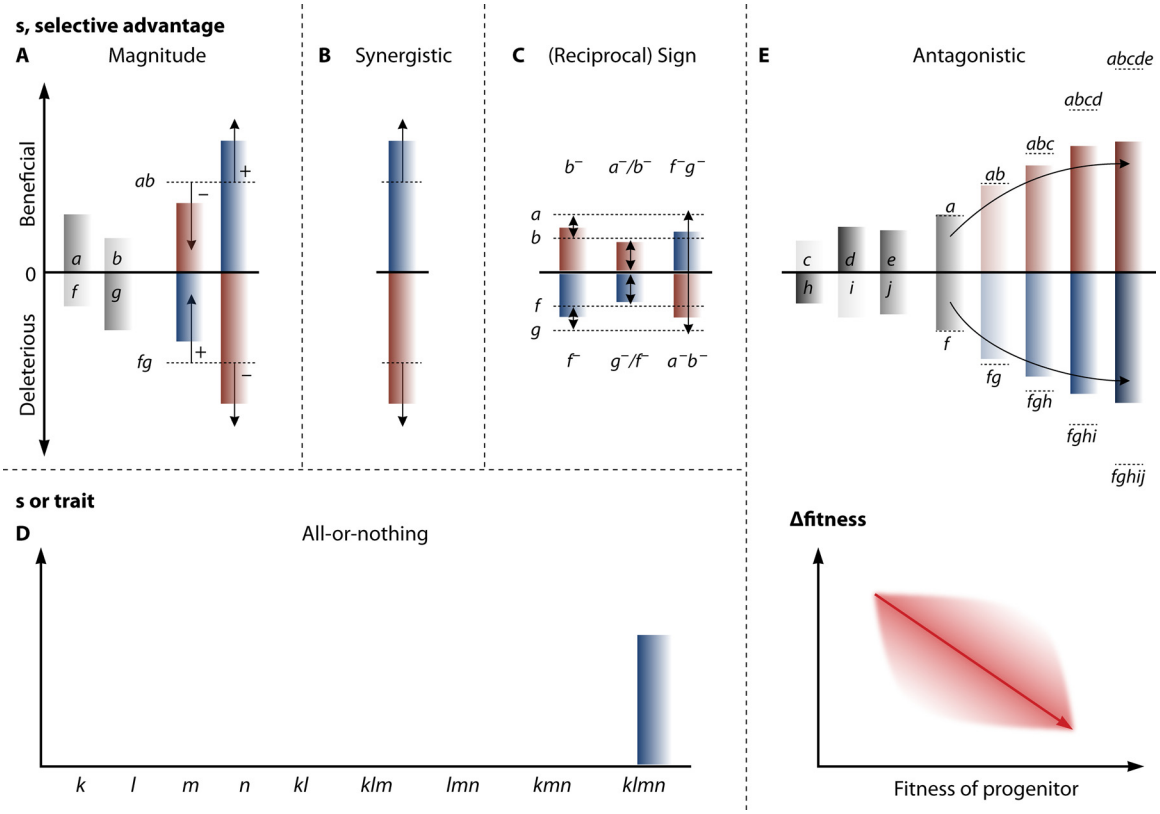

FIG 3 Overview of the possible forms of genetic interactions among mutations. (A) Genetic interactions are either negative (red) or positive (blue) forms of magnitude epistasis if the combined effect is either smaller or larger than the expectation under an independent interaction (dashed lines and letters). Outcomes and their color classification are shown for the combination of 2 single beneficial (*a* and *b*) or deleterious (*f* and *g*) mutations (gray bars). Note that the color for magnitude epistasis and the indication of the expected effect under independence are used throughout the figure. (B) Under synergistic epistasis, two beneficial or deleterious mutations enforce each other in their combination, leading to a more extreme effect. Synergistic epistasis between beneficial mutations would be classified as a positive interaction (blue), while that between deleterious ones is negative epistasis (red). (C) Sign epistasis constitutes the transformation of one or both beneficial mutations to become deleterious (negative magnitude epistasis) (red) or deleterious mutations to become beneficial (positive epistasis) (blue) due to their combination. In our example, if the combined fitness effect of mutations *a* and *b* falls below the single effect of mutation *a* but is higher than the single effect of mutation *b*, only mutation *b* is affected by sign epistasis, and mutation *a* remains beneficial. If the fitness of the double mutant is lower than that of mutation *b* only, it is impossible to distinguish whether mutation *a* or *b* is influenced by sign epistasis. However, they cannot both be affected by sign epistasis. If both mutations *a* and *b* changed signs, the combined mutants will show deleterious fitness altogether, which is called reciprocal sign epistasis. (D) In the case of all-or-none epistasis, multiple mutations cooperate to generate a fitness effect or the emergence of a trait. Moreover, they are all required. If any of the mutations is missing, the effect of the trait is not expressed. (E, top) Antagonistic epistasis involves interactions between multiple beneficial or deleterious mutations. For beneficials, it can be viewed as negative epistasis (but not sign epistasis) that is enhanced with each additional mutation (red), and vice versa for deleterious mutations (blue). While double mutant *ab* or *fg* still closely corresponds to the expected effect without interaction, the distance under neutral conditions increases for triple mutants *abc* and *fgh* and even more so for quadruple mutants *abcd* and *fghi* and quintuple mutants *abcde* and *fghij*. (Bottom) Antagonistic epistasis is widespread, especially for beneficials, and can be generalized to a condition of diminishing returns, where the effect of the beneficial mutation decreases with the fitness of the progenitor in which it arises.

maladapted derivatives of a general ancestor showed the most fitness progression over time and succeeded in catching up with populations that started from more-adapted founders (259). A correlation between initial fitness and adaptivity has been seen by others as well (Fig. 3E) (317, 321–323). The different adaptation rates mainly correlated with the fitness of the founder, not its genotype, and were the result not of a differential availability of possible beneficial mutations but of a global pattern of diminishing-returns epistasis among beneficial mutations (259). As such, the effect of the beneficial mutations decreased with increasing background fitness. The same is true for the LTEE, where the effect of each of the first few mutations that were fixed in the Ara-1 population proportionally declined with the increasing number of remaining mutations in (and, thus, fitness of) the background (240), which was recently extrapolated to be true when constructed in a more diverse set of natural isolates (324) or

when the LTEE was studied over a longer time scale with replay experiments (325). At roughly the same time and in a similar way as the study described above (240), the prevalence of diminishing-returns epistasis between adaptive mutations and its effect on the rate of adaptation were also reported for the experimental evolution of *M. extorquens* populations that grew on methanol based on a native engineered pathway (318).

Synergistic epistasis. In synergistic epistasis, mutations tend to enhance each other's effects (Fig. 3B). Thus, a mutant with two beneficial mutations will have an improved fitness that is higher than expected based on the sum of the effects of the individual mutations. Just like sign epistasis, this form of genetic interaction is not very common among fixed mutations in experimental evolution (240, 259, 318).

During adaptation to glycerol, for example, *E. coli* almost always acquired at least two mutations, one in the RNA polymerase and another in *glpK*, the gene coding for glycerol kinase (254). Their frequent cooccurrence is explained by their synergistic interaction that improves metabolic efficiency and growth on glycerol (309, 326). A similar example can be found in the adaptation of *E. coli* to lactate, where the effect of an 82-bp deletion in *rph-pyrE* was increased in a variety of genotypes that already carried mutations adaptive to lactate limitations, which also explains why it is often present in independently evolved populations (258). In the LTEE, the benefit of a mutation in *pykF* in the Ara-1 population increases as the background acquires more beneficial mutations and thus becomes more adapted to the LTEE environment (240). Across multiple populations of the LTEE, for multiple beneficial mutations that were fixed in *pykF* during the LTEE and over the course of 50,000 generations, epistasis is, however, more complex and dynamic, combining different forms of genetic interactions (327). Also, in adaptation to isobutanol, some of the adaptive mutations in the endpoints interact synergistically (137, 139). While most of the cohort mutations arising in *S. cerevisiae* populations growing in glucose-rich medium contained only single driving mutations, one cohort could expand due to the synergistic interaction between two mutations located in previously unidentified targets of evolution under these conditions (299). A synergistic epistatic interaction detected during experimental evolution can also have profound medical consequences. The evolution of *S. aureus* toward high-level resistance to colistin, a last-resort antibiotic, was determined by strong synergistic interactions among 5 contributing mutations (328).

Although all of the above-described scenarios are examples of interactions between beneficial mutations, similarly, two deleterious mutations can combine into a double mutant with an even more negative effect than expected (Fig. 3B). Out of six mutations identified to result in trimethoprim resistance, two are actually deleterious in a background already containing the four other mutations (sign epistasis). Combining them into a sextuple mutant further aggravates their separate effects and results in a mutant with lower resistance than expected because of synergistic interactions between the two mutations (278). An MA experiment in yeast suggested generalized synergistic epistasis among deleterious mutations since the rate of fitness decrease increased in time (329). Although others have made similar observations (330), the opposite observation has also been made (61, 331, 332) (an example of antagonistic epistasis), and clearly, not all combinations of random deleterious mutations show synergism, making a generally synergistic epistatic interaction among deleterious mutations questionable (333–335).

Sign epistasis. An extreme example of genetic interaction is sign epistasis (Fig. 3C). In this case, the effect of a beneficial or deleterious mutation becomes negative or positive, respectively, when arising in an alternative background. Although this kind of interaction is not frequently encountered in fixed mutations in experimental evolution (240, 259, 318), the cases that show sign epistasis have a profound impact on the dynamics and outcomes of evolution.

In the Ara-1 populations of the LTEE, a mutation in *ompF* was found to have a deleterious effect when introduced into the ancestral strain (238). Still, this mutation was able to be fixed in these populations, a result of competition with other mutated

alleles of *ompF* that contended for fixation (CI). So while deleterious on its own, the *ompF* mutation was in fact beneficial in a more adapted background (15). Similarly, the stable coexistence of two ecotypes in the LTEE required the fixation of two mutations (in *arcA* and *gntR*) that were neutral and deleterious, respectively, when introduced into the ancestral background (see "Sustained diversity from negative frequency-dependent selection," below). Their fixation was possible only by sign epistasis because of earlier fixed mutations (336). In yeast strains adapting to glucose-limiting conditions, reciprocal sign epistasis between two adaptive mutations transformed them in deleterious ones and made them mutually exclusive. As a result, reciprocal sign epistasis explains the separation of two distinct peaks by a valley in a rugged fitness landscape (255). During adaptation to the fungicide nystatin, reciprocal sign epistasis was more widespread and affected one-third of the pairwise combinations of first-step mutations (337). Similar reciprocal sign epistasis between fixing mutations explains the rugged fitness landscape on which *E. coli* evolves under glucose-limiting conditions in a chemostat (315) and when adapting to increasing ethanol concentrations (338). An extreme example of reciprocal sign epistasis can be found when two beneficial mutations combine into a double mutant that is lethal. Synthetic lethality can also occur as a specific case of synergistic epistasis between two deleterious mutations or as all-or-none epistasis (see below) between two neutral mutations. Synthetic lethality is most often reported when constructing artificial double mutants (332, 339), and it is rarely detected between mutations identified in evolving microbes in the laboratory. Most reports come from evolution experiments with viruses, where synthetic lethality in tobacco etch virus (TEV) adapting to a new host resulted in holes in the fitness landscape (340) and genetic incompatibility between mutations leading to OmpF and LamB specialists in phage lambda resulted in the first step of speciation (341). As such, synthetic lethality could more generally also underlie the mutual exclusion of beneficial mutations (338).

The possible constraints of sign epistasis on evolution are very clear in β-lactam resistance conferred by five mutations in a certain β-lactamase. Although 120 theoretical paths to reach the quintuple mutant with the highest fitness are possible, only 18 of them are adaptive (342, 343). In contrast, the fitness landscape of evolution toward trimethoprim resistance revealed more-indirect paths when also considering high-order (sign) epistasis, taking into account that the kind of interaction between a pair of mutations can depend on the presence or absence of any combination of the additional mutations. Some adaptive paths resulted in a loss of a certain mutation, which delayed the commitment to a genotypic fate (278). So far, the effects of these high-order epistatic interactions on evolutionary dynamics have been understudied, but they have been shown to be equally as important as pairwise interactions in tobacco etch virus adaptation to new hosts as well (340). On a similar note, the cost of some antibiotic resistance-conferring mutations under drug-free conditions can be completely reverted by yet another resistance-conferring mutation or can be highly dependent on the genetic background in which it appears (344–346). Even without being specifically measured, sign epistatic interactions likely also play a role in the historical contingency of adaptation of resistant *P. aeruginosa* mutants to new antibiotics. Indeed, some initial adaptive trajectories to resistance to the first drug limited subsequent evolution of resistance to the second, while evolution of resistance to the second antibiotic can also revert the initial resistance (28).

A more abundant form of sign epistasis potentially remains hidden in evolution experiments. Different adaptive mutations in the same gene are hardly ever fixed in the same background, very likely because the presence of a first beneficial mutation makes subsequent adaptive mutations neutral or deleterious (110, 224, 319). Indeed, except for some notable examples of intergene sign epistasis (332), sign epistasis is believed to be more prevalent between adaptive mutations in the same gene (342, 347–349).

All-or-none epistasis at the basis of innovations. In evolution experiments in simple and constant environments, the fitness trajectory usually resembles one of gradual improvement, which is in line with the stepwise process of Darwinian theory (1)

and probably results from widespread diminishing-returns epistasis, as discussed above. Nevertheless, more-sudden fitness improvements also occur, and these can be explained by all-or-none epistasis (Fig. 3D). Here, a combination of different mutations is necessary for the abrupt fitness increase, while, as long as one of the mutations is missing, the others have no effect on fitness. The accumulation of multiple strictly neutral mutations in the "right combination" is possible but unlikely, and for now, the strict formulation of this kind of interaction in terms of its fitness effect has, to our knowledge, not been observed.

All-or-none epistasis still remains a valid explanation for the emergence of some innovative phenotypes during evolution experiments. In this case, initial mutations (neutral or beneficial mutations) would potentiate the emergence of a driver mutation that actually creates the new phenotype. None of the potentiating mutations show any form of this phenotype prior to actualization, but without these mutations, the driver mutation would be futile, having no or little effect (on fitness and/or the innovative phenotype) and no chance to be fixed at all. Later, the new phenotype could be further optimized in a refinement period through additional mutations.

This process of potentiation-actualization-refinement was first observed when the Ara-3 population of the LTEE evolved the capacity to grow on citrate under oxic conditions after 31,500 generations (13), caused by a rare driver amplification that made a preexisting citrate transporter oxygen responsive because of a promoter capture event (241). During the first 15,000 generations, the population accumulated mutations that created the tendency to evolve a Cit$^+$ phenotype (13), while additional mutations after its actualization further refined this key innovation (241). Since the actualizing mutation and one of its refinement mutations were sufficient for growth on citrate, the qualification as all-or-none epistasis was somewhat questioned (350), although potentiating periods where necessary mutations presumably accumulated have also been reported for the fast emergence of Cit$^+$ phenotypes during direct selection schemes (351). It thus seems very likely that the potentiating mutations made the very rare driver mutation (more) beneficial, and thus, at least at some point in the evolution, the Cit$^+$ phenotype was dependent on its history by all-or-none epistatic interactions (13). In the usage of another carbon source by *E. coli*, ethylene glycol, all-or-none epistatic interactions were present between 2 mutational events. Similar to the emergence of citrate utilization, the acquirement of the actualization mutation allowing adaptation to ethylene glycol was also historically contingent on a previous mutation, selected by adaptation toward propylene glycol usage (352). In a similar way, all-or-none epistasis was responsible for the *de novo* evolution of stochastic switching between two different phenotypes in *P. fluorescens*. Here, a total of nine mutations was found, and while the last was necessary and also sufficient to cause stochastic switching, the previous eight were necessary to set the stage by improving its fitness effect (25). In *Salmonella enterica*, new genes were shown to evolve but only after an all-or-none epistatic type of innovation in a preexisting gene. In medium lacking histidine and tryptophan and in a mutant missing a key enzyme in the production of tryptophan, TrpF, *hisA* needs to acquire two mutations to take over the TrpF activity while maintaining its native function in histidine production (353). Afterwards, by the processes of amplification and divergence, new genes with segregated functions arose (a process called subfunctionalization [354]). Another innovation by all-or-none epistasis arose during the coevolution of *E. coli* with phage lambda, where the phage evolved the capacity to use an alternative receptor, OmpF instead of LamB, to infect *E. coli*. A single mutation enabled the use of OmpF, but the effect of this mutation was dependent on the presence of three others, which improved attachment to the ancestral receptor LamB, and on the coevolution dynamics of the host toward resistance to the effect of those infectivity-improving mutations (355, 356). A human pathogen, avian influenza A/H5N1 virus, gained the capability of airborne transmission in the laboratory (23) by the combination of five mutations (357), some of which were already present in natural populations (358). Finally, multicellularity, a major transition in the history of life itself, evolved in yeast by the combination of several mutations that

were all necessary. Here, the low sucrose concentration in the medium used made multicellularity a beneficial trait because of the obligate extracellular digestion of sucrose before it could be used as a nutrient (359).

In contrast, other transitions from unicellular life to a multicellular state in experimental evolution might not have emerged from all-or-none epistatic interactions (129, 360, 361), for example, when *E. coli* starts to aggregative strongly during the evolution of a passive mutualistic interaction involving the exchange of amino acids (362), in the origin of simple multicellularity in *Chlamydomonas reinhardtii* to escape predation (363), or in a setup favoring faster settling (364). In *S. cerevisiae* genotypes that show fast sedimentation in clusters, only one frameshift mutation was sufficient to cause the multicellular trait (365). Other innovations are also less dependent on the collaboration between different mutations and do not rely on an all-or-none interaction exhibited in the above-described examples. Superior social cooperation, for example, can evolve in a population of obligate cheaters in the spore-forming bacterium *Myxococcus xanthus* by acquiring just a single mutation (253, 366), and in the same bacterium, *de novo* kin discrimination evolved gradually over time by multiple mutations or all of a sudden but probably by a single mutation (367). Likewise, flagellar locomotion could be fully resurrected in a *P. fluorescens* strain made immotile due to a deletion of the main regulator of flagellar synthesis, by only two mutations that each gradually increased motility (368).

Natural Selection for Suboptimality

It is a common misapprehension that evolution always leads to improvements and survival of the fittest. Clearly, in the small populations of MA experiments, natural selection is not strong enough, and the outcome will be of a degenerate nature. However, even under strong natural selection, in large populations where drift and stochastic processes are being outperformed by adaptation, the fittest individuals do not always prevail (Fig. 4).

Second-order selection. As stated above and because of CI, an individual that is unfit at present could outperform the currently fittest individual if it can acquire additional beneficial mutations and hence become more fit. As a result, selection in evolution experiments not only acts on direct fitness but also can contain an extra layer, i.e., second-order selection for more-evolvable individuals (Fig. 4A).

In the Ara-1 population of the LTEE, for example, second-order selection for greater evolutionary potential selected eventual winner genotypes that were, at an intermediate time point, less fit than eventual losers but prevailed nevertheless because of more-beneficial epistatic interactions with subsequent mutations (369). Similarly, in *E. coli* evolving in a chemostat under glucose limitation, a duplication was fixed and remained stable, not because of its own effect on fitness but probably because of its higher evolutionary potential (370).

Second-order selection could also be an explanation for the frequent emergence of hypermutators in experimental evolution and natural populations or for the existence of a stress-inducible form of mutagenesis (SIM) (38, 67, 113, 128, 371, 372). The increased mutation rate would result in a higher probability of producing a rare beneficial mutation or a combination of multiple ones, which is likely important under complex selection pressures. Subsequently, the mutator or SIM genotype could hitchhike along with the beneficial mutation. Despite the direct risk of increasing the genetic load and decreasing fitness by the higher statistical chances for the mutated offspring to accumulate deleterious mutations rather than hitting a beneficial one, such an increase in evolvability can still be indirectly beneficial to a population (197, 373). A mutator in *Candida glabrata*, for example, has been shown to accelerate the evolution of caspofungin resistance in a mouse infection model (374). Likewise, the emergence of mutators was key in the adaptation of *E. coli* toward high levels of ethanol tolerance, where they reoccurred during each adaptive step but were compensated for as soon as adaptation was reached (65). Similarly, a transient state of hypermutation was necessary to accelerate the adaptation of *Ralstonia solanacearum* to its new endosym-

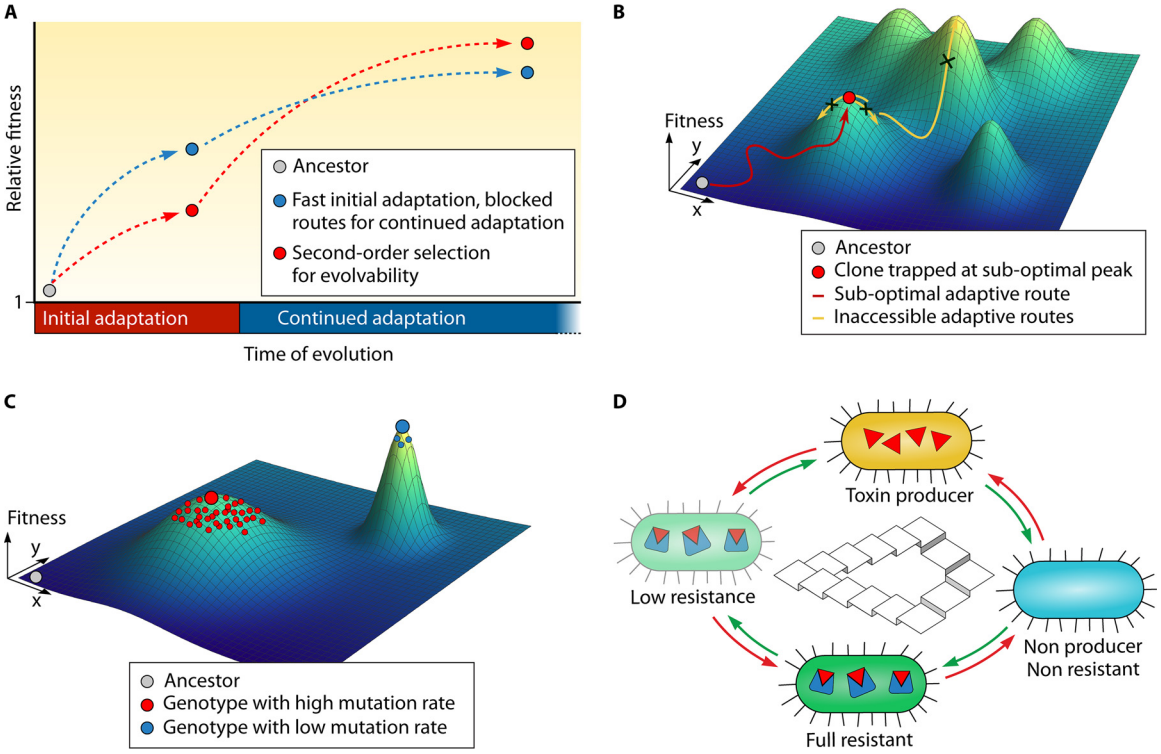

FIG 4 Evolution of suboptimality. (A) Second-order selection takes into account not only a genotype's current fitness but also its evolvability in the future. As a result, genotypes that are outcompeted by superior mutants can be selected, as long as they prevail in the end. (Based on data from reference 369.) (B) On a rugged fitness landscape, a more-optimal peak might simply not be accessible because the path toward it involves decreasing fitness first. If crossing such a valley is impossible, genotypes are stuck at a suboptimal peak. (C) Depending on the mutation rate and the population size, populations of a genotype might in fact be highly diverse, e.g., viral populations. Under these conditions, the overall population fitness strongly depends not only on the height of the fitness landscape at the consensus genotype but also on the topology in close proximity. Sharp peaks might therefore be disadvantageous to highly evolvable species, while flat peaks are preferred, even when their fitness is lower. (D) Nontransitive fitness effects lead to dynamics best described by the illusion of a Penrose staircase, whereby an immediate successor always outperforms its immediate predecessor (green and red arrows), but evolution eventually results in a genotype that can again be outcompeted by the ancestor. As an example, a rock-paper-scissor game between toxin producers, resistant types, and nonproducer, nonresistant strains is depicted on the stairs.

biotic role in the rhizosphere of *Mimosa pudica* (375). Although it is suggested that this is especially the case under stressful conditions that require quick adaptation or difficult (combinations of) mutations (65, 128, 371, 376), an increased mutation frequency has also been found in the simplest evolution experiments, like the LTEE (228). Here, mutators are likely beneficial as long as the population is not overly well adapted so that they have great potential to hitchhike to fixation on the beneficial mutations that mutators generate at an increased rate. After the Cit$^+$ phenotype evolved and there was great potential for improvement, mutators emerged strikingly quickly (64, 197, 241, 377). The second-order selection rationale remains controversial, as the reasons for the existence of SIM or the emergence of mutators are far more complex, and other explanations exist as well, e.g., drift, the pleiotropic by-product of adaptation, or a shifting fitness landscape under altered environments that results in a DFE more biased toward beneficial mutations (378–380). More-in-depth overviews can be found elsewhere (380–382).

Suboptimal peaks and selection of the flattest. Evolution by natural selection does not necessarily lead to the highest peak in the fitness landscape. In rugged landscapes, for example, an adaptive walk might "miss a turn" and get stuck at a suboptimal peak surrounded by valleys of maladapted genotypes that are impossible to cross (Fig. 4B) (383, 384). So far, only very limited empirical data exist to show replicate populations trapped at such evolutionary dead ends (65, 255).

Alternatively, suboptimal peaks surrounded by less-maladapted variants can be

preferred over more-optimal solutions that have highly maladapted variants in their direct neighborhood as a kind of genetic robustness and "selection of the flattest" (Fig. 4C) (385, 386). Especially when mutation rates are high in large populations, as shown in competition experiments with RNA viruses, the genotype with the highest fitness wins under normal conditions but loses when the mutation rate is artificially increased (387).

Nontransitive fitness and the Penrose staircase. Relative competitive fitness can be nontransitive, e.g., when an endpoint outcompetes its immediate predecessor but has a lower fitness than that at an earlier time point. As such, fitness landscapes might not contain an absolute peak (14, 388), and it is thus possible that the fitness landscape looks more like the illusion of the Penrose staircase, where an immediate successor is always more fit than its predecessor, just like steps in the Penrose staircase that seem to perpetually ascend in a continuous loop (Fig. 4D). Nevertheless, the ancestor can outcompete, or at least be equally as competitive as, more-distant successors, so it appears as if evolution went full circle, in analogy to climbing the Penrose stairs, where you end up at the place where you started, without getting higher.

Nontransitiveness of fitness has been observed in certain evolution experiments and indeed even caused an apparent loss of fitness compared to that at the starting point (25, 218), but it is far from a general rule. For example, it was not observed over the long time scale of the LTEE (389), and fitness is likely nontransitive only if special social interactions are present (316). For example, consider the artificial system with three *E. coli* strains where one produces toxin, the second is toxin resistant, and the third is neither resistant nor a producer. Since the second tops the first, the third outperforms the second, and the first outcompetes the third, competitive fitness is clearly nontransitive and could result in a rock-paper-scissors game (Fig. 4D) (390). In a similar way, loner strains of *P. aeruginosa* stabilize the community of cooperators and defectors in a social mechanism to take up iron by inducing rock-paper-scissors dynamics. Also, in experimental coevolution between *P. fluorescens* and its parasitic phage SBW25Φ6, after an initial period of arms race dynamics (ARD), evolution becomes less directional and is dominated by fluctuating selection dynamics (FSD). During the initial period of ARD, fitness increases progressively by increases in mean infectivity and resistance in phage and host, respectively, in response to each other. Under FSD, however, alternative hosts and parasites are continuously selected based not on continuously increasing fitness anymore but on differences in specific infection or resistance mechanisms (172, 391).

SELECTION FOR SPECIALISTS OR GENERALISTS?

Many evolution experiments keep conditions constant. Whereas small changes in growth conditions are encountered in each cycle in serial transfer experiments, chemostats maintain identical conditions over time. Adaption to these conditions (and conditions where stress levels are progressively increased) is often believed to select for specialists that are tailored to their specific environment but show trade-offs in other, novel environments, also called negative correlated responses or the "cost of adaptation." These trade-offs likely also contributed to the evolution of diversity in nature (see Diversity in Experimental Evolution, below). In certain cases, adaptation leads to no or small trade-offs or comes with positively correlated responses, which pave the way for more-generalist genotypes.

Pervasive Trade-Offs and Specialists in Experimental Evolution

Trade-offs costs are often observed in experimental evolution (Fig. 5A) (161, 194, 360). Viruses are well-known specialists with respect to either their eukaryotic or prokaryotic hosts (392). During adaptation to a new host, infectivity usually decreases in the original host (124, 152, 165–167, 175, 251). In phage lambda, while initially selected as a generalist to be able to infect both LamB- and OmpF-expressing hosts (355), further adaptation to both single hosts led not only to extreme specialization events but also to an initial speciation event whereby specialists became reproductively

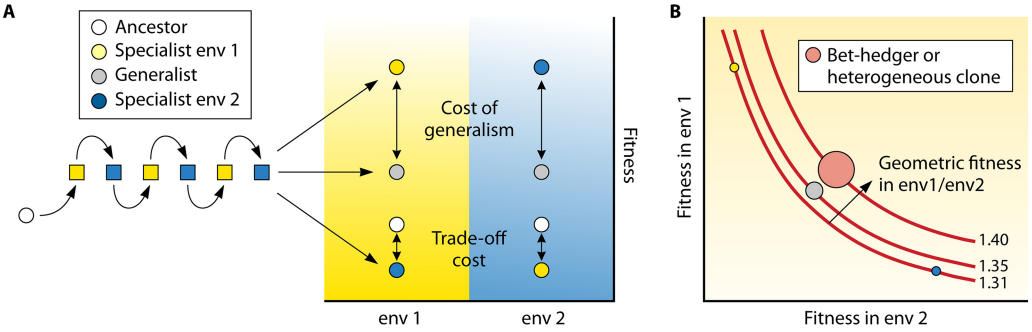

FIG 5 Emergence of specialists, generalists, or bet-hedgers. (A) When an ancestor encounters 2 new environments (env), it can specialize to either of them, often at a trade-off cost in the other environment. Alternatively, adaptation can lead to a generalist that is often worse than any of the specialists in their preferred environments (cost of generalism) but has the advantage of minimizing fitness fluctuations across both environments. As a result, generalists are often selected for under changing conditions. (B) Under certain conditions, the geometric fitness average across changing environments can be further optimized in a bet-hedger or a clone that shows population heterogeneity, thereby effectively combining individuals from both specialist classes. Consider the specific example where specialists are successful under one condition (fitness of 1.9) but maladapted under the other (fitness of 0.9), a generalist that performs equally well under both conditions (fitness of 1.35), and a bet-hedging strain with two variant cell types of equal frequencies and each considered to be a specialist under one condition. The fitness under each separate condition is lowest for the generalist (fitness of 1.3), followed by the bet-hedger (fitness of 1.4), all to be outcompeted by each specialist (fitness of 1.9). However, when conditions alternate but remain equal in frequency and duration, the generalist outcompetes both specialists, as its geometric mean fitness is higher: 1.35 versus 1.31 for the specialists ([rad,2]1.9×0.9). While their arithmetic mean fitnesses are equal (fitness of 1.4), the bet-hedger also outcompetes the specialists, with a geometric mean fitness of 1.4 ([rad,2]$(0.5 \times 1.9 + 0.5 \times 0.9) \times (0.5 \times 0.9 + 0.5 \times 1.9)$), thereby even outperforming the generalist. Sizes of the circles are scaled to their geometric fitness, which is also shown by the curved lines and numbers.

isolated and genetically incompatible (341). When selecting for delayed transmission times, extracellular survival improved, but viral fecundity decreased (393), while adaptation to a high multiplicity of infection resulted in lower success at a low multiplicity of infection (394). Adaptation of plasmids highly resembles the evolution of viruses, with trade-offs toward hosts that were not encountered during adaptive evolution (395, 396). The LTEE displayed an extreme example of specific trade-off by specialism. Here, the optimal growth of adapted mutants on glucose not only depended on citrate as iron chelator (397) but also was specialized to the temperature used (233). Most trade-offs in experimental evolution are found either in the use of alternative nutrient sources or at different concentrations (37, 232, 359, 398, 399), but also, when evolving microorganisms to more-stressful conditions, trade-offs are widespread (100, 111, 139, 148, 400). Evolution of resistance toward antibiotics, viruses, or predators often bears trade-offs, not only as a cost under drug- or predator-free conditions (169, 401) but sometimes also in the form of hypersensitivity to other drugs. For antibiotics, trade-offs mainly exist between two main groups, aminoglycosides and all the other classes of antibiotics (114, 130, 402). In the case of evolving thermal specialists, trade-offs at off-target temperatures often arise: the further from the selected target temperature, the stronger the trade-off, while in close proximity to the thermal optimum, positive pleiotropy can be observed (108, 141, 142, 153). For *E. coli*, specialization to thermal niches was brought down to the genotype, as more overlap was detected in the mutated genes between replicas of the same treatment than in the intertreatment comparison (403).

These trade-offs can result from the accumulation of neutral mutations in the selective environment that become deleterious in newly encountered situations, the MA effect (399). Alternatively, trade-offs can arise because of antagonistic pleiotropy (AP) (also called adaptive trade-offs), the process where adaptive mutations under one condition are maladaptive under another (227). The latter could, for example, be the result of mutual exclusiveness between the mechanisms that are selected for and therefore between different solutions to the selective pressures under both (often contrasting) conditions. Whether solutions are really mutually exclusive and trade-off costs cannot be compensated for without the loss of the fitness effect of the original

optimized mechanism is, however, not often examined in great detail and therefore is not discussed in this review. The relative contribution of either MA or AP to trade-offs is still a matter of debate and could be different depending on the conditions. Clearly, the two mechanisms do not need to be mutually exclusive. On short evolutionary time scales, the accumulation of neutral mutations that are deleterious under novel conditions seems less likely to explain the origin of trade-offs (except when mutators arise) than beneficial mutations with antagonistic pleiotropic costs (194, 232, 359, 400) or than epistatic effects between multiple mutations that change depending on the environment (242, 404, 405). However, trade-off costs in *Chlamydomonas* evolving in the light or the dark or metabolic erosion during the LTEE in *E. coli* under glucose-limited conditions or in *Saccharomyces paradoxus* during adaptation to different carbon sources are explained mainly through gradual mutation accumulation (162, 399, 406).

Although trade-offs clearly are a general feature of evolution experiments, they are not universal. Positively correlated responses have been observed, a form of synergistic or positive pleiotropy (153, 189). For example, evolution on a single carbon source can also lead to the improved use of other naive carbon sources that were not encountered during the experiment (398, 399, 407). Also, more-complex situations might arise, such as during evolution on glucose, which did not improve the direct performance of *E. coli* on lactose as a sole carbon source but improved its evolvability for adaptation using lactose (408). Although antibiotic resistance normally carries a cost under drug-free conditions (409), the evolution of *E. coli* at 42.2°C synergistically led to resistance to rifampin (410). Evolution in the presence of one antibiotic often also results in resistance to other antibiotics (cross-resistance) (130, 131). Likewise, the evolution of methicillin-resistant *Staphylococcus aureus* (MRSA) in biofilms sequentially selected for a bacteriocin-excreting mutant outcompeting the ancestor, followed by the emergence of a mutant resistant to the bacteriocin, which also showed resistance to the last-resort antibiotic vancomycin (411). This kind of cross-resistance also evolved under more-general stress conditions with radiation, osmotic, acidic, oxidative, or solvent stress (101, 111).

Generalists in Changing Environments

If trade-offs between adaptations to different conditions are inevitable and strong and the environment changes too infrequently between these conditions, evolution can lead to the sequential selection of condition-specific specialists (25, 172, 412, 413) (see "Clonal interference and soft sweeps," below). If not, a changing environment is predicted to select for generalists, instead of specialists, that do well under all separate conditions, a "Jack-of-all-trades" (Fig. 5A), which is often true for coevolution between predator and prey. Because of the ARD under the RQH, both partners evolve to become generally better at infecting and resisting, respectively (173, 175), although continued evolution and heterogeneous conditions with more than one predator and prey could lead to FSD or the coexistence of different specialized genotypes (see "Mixed environments with heterogeneity in niches," below).

Cost of generalism. As foretold by the phrase "Jack-of-all-trades, master of none," the evolution of generalism can be constrained since trade-offs can impair the extent or speed of evolution. An alternative explanation for the constrained evolution of generalists is the shorter time that each separate condition is experienced when selecting for generalists by changing environments, which should be accounted for when comparing results of evolution under changing and constant environments. The resulting lower fitness of the generalist under each separate condition than of the condition-specific specialist has been called the cost of generalism (Fig. 5A) (157).

This was clearly the case during evolution alternating between high and low oxygen concentrations (107), temperature extremes (159, 414, 415), antibiotics (130, 416), or hosts in the case of viruses (126, 165). The most extreme example is when environments fluctuate randomly between different kinds of selection pressures. The fitness of *E. coli* populations under these fluctuating conditions improved only marginally under the separate acid, osmotic, and oxidative stress conditions that were used (similar to

experimental evolution under random temperatures chosen between two extremes [189]). Instead, their performance improved when challenged by novel, unexpected stresses (134). While extending the original evolution experiment in time and to more replicates showed improvements in the mean fitness under the separate stress conditions, it foremost resulted in a total loss of trade-off costs and decreased variance in fitness across environments, showing signs of the appearance of true generalists (417).

Still, in all these cases, the "Jack-of-all-trades, master of none" is selected over specialists, as it minimizes fitness fluctuations over time (as in the extended phrase, "Jack-of all-trades, master of none, often times better than a master of one") by adapting not only to all the separate conditions but also to fluctuations as such. As a result, measurements made with the separate, constant components were shown to be bad predictors of the level of adaptation to the fluctuating environment itself (160).

Cost-free and superior generalism. Since side effects are not universal and the simple accumulation of mutations might be the underlying factor for trade-offs, cost-free generalists could emerge during adaptation to a fluctuating environment. Cost-free generalism would imply that fitness under each separate condition is comparable to the fitness of specialists that evolved under separate constant conditions ("Jack-of-all-trades, master of at least some"). Indeed, such cost-free generalists have often been observed (155), for example, in the evolution of the photosynthetic *Chlamydomonas* species in cycles between dark and light (162, 163), in some reports on viruses that alternated between different host cells (152, 167), or in the adaptation of *E. coli* to alternations between acid and alkaline conditions (158). Generalists are sometimes superior to the condition-specific specialists ("Jack-of-all-trades, master of all"), as observed in the evolution of vesicular stomatitis virus to fluctuating temperatures (189) or when chikungunya virus adapted to alternating hosts (166).

A recent report on evolution in the presence of fluctuating levels of glucose or lactose, however, showed that although an initial period of adaptation might be one of cost-free generalism, at longer time scales, trade-offs that constrain either the speed or the extent of evolution become inevitable (182). The authors of that report explained the somewhat counterintuitiveness of cost-free and superior generalism by the small number of generations during many evolution experiments. In contrast, pleiotropy in epistatic interactions could lift certain evolutionary constraints and open up superordinate paths that are inaccessible to adaptation in both constant environments, which would be another explanation for cost-free generalism.

Evolution of the bet-hedger, the specialist-generalist, and the importance of clonal phenotypic heterogeneity in evolution. Nature has an alternative answer to fluctuating environments besides evolving generalists or specialists, which could be regarded as genotypes that simply sense and respond, at a population-wide level, to changes in the environment. We then consider a generalist to be a genotype that can sense and respond to all condition shifts, while specialists are responsive to only one (418). Bet-hedging is a kind of phenotypic multistability in an isogenic population where the individuals present one of different phenotypes, each specialized for a certain condition but often less fit for other conditions (419). This heterogeneity by the combination of phenotypic specialists can be seen as a special form of generalism (i.e., a combination of many "Jacks-of-one-trade"). It might decrease the average fitness of the population because of the presence of suboptimal variants but instead can minimize the variance of fitness over time; i.e., it can maximize the geometric mean fitness or long-term fitness of a population in changing environments and partially avoid the cost of generalism that generalists could suffer (Fig. 5B) (420). The geometric mean is indeed a better measure for long-term success than the arithmetic mean, intuitively, as selection over many generations is a multiplicative process (384, 419), but also theoretically, since fitness over time under changing conditions is not an independent linear combination of each separate fitness but rather the result of an interacting combination. For example, when part of the population is lost under one condition, this part cannot generate offspring under the next, beneficial condition.

Bet-hedging is predicted to be advantageous over specialism or normal generalism

involving sense-and-response systems under some conditions where environments change infrequently (421, 422), changes are hard to predict and to sense (422, 423), and/or changes are extreme (strong trade-offs) (423, 424). The configuration of bet-hedging (i.e., the number of different phenotypes and the rate of switching between them) needs to maximize the geometric mean fitness and therefore should be proportional to the environmental dynamics, e.g., the frequency of each condition, the rate of alternation between them, and the fitness of each variant in each of them (Fig. 5B) (421–423, 425, 426).

Although bet-hedging is widespread in nature and many observations seem to support the theoretical predictions listed above (425, 427–431), reports of experimental evolution dealing with bet-hedging behavior remain scarce. Periodic selection for competence in *Bacillus subtilis* maintained bet-hedging (432), while evolution in a constant environment lacking selection for sporulation led to the loss of bet-hedging behavior (433). The latter was because of mutation accumulation rather than selection, but the *de novo* evolution of a bet-hedging phenotype in *P. fluorescens* under conditions alternating between shaking and nonshaking environments provided stronger proof for the adaptive nature of bet-hedging (25). Under each of these conditions, genotypes with different colony morphotypes usually evolve, but by imposing bottlenecks and exclusion rules, i.e., transferring only a single colony of a novel morphotype as soon as it arose, to the alternative environment, a genotype that phenotypically switched between morphotypes emerged (25). These bottlenecks and exclusion rules are analogous to sudden and extreme environmental changes: the fitness of the ancestral morphotype is arbitrarily set to a value of zero, and the cost of generalism by bet-hedging is held low by limiting competition. Moreover, they were an absolute prerequisite for the evolution of bet-hedging in this case and likely operate in many natural environments (424). The configuration of bet-hedging was also shown to experimentally evolve according to the environmental dynamics. Dormancy levels in the fungus *Neurospora crassa*, for example, were shown to evolve according to the frequency of bad years (434). Likewise, the number of phenotypic antibiotic-tolerant cells in a population of many bacterial species can be increased to extremely high levels by frequent antibiotic treatments (26, 27, 164, 435). The adaptive process under these conditions seems to be even more specific, as the rate of switching of tolerant *E. coli* cells back to the normal cell type was shown to evolve according to the treatment duration (26), while the rate of switching of normal cells to antibiotic-tolerant persisters evolved according to the imposed treatment frequency (27). The evolution of antibiotic bet-hedging strategies can have profound consequences on future evolution dynamics, impacting our medical care. Indeed, these persisters have been shown to be the first stepping stone to the evolution of resistance (436), also in evolution experiments with tumor populations (437, 438). Under some conditions, it seems that the initial evolutionary response to strengthen tolerance proceeds and opens the paths to the evolution of resistance development and thereby accelerates it (439). Here, this increased evolvability likely is a combined result of a game of numbers (more survival and larger populations) and increased mutation rates, although epistatic interactions cannot be excluded. Similarly, clonal heterogeneity and stochastic expression of antifungal resistance genes increased the evolutionary potential with gradually increasing concentrations of the drug (440). This increased potential likely stems from a modulation of the fitness benefit of beneficial mutations. The single-cell lag times of switching between carbon sources in yeast have also been shown to be heterogeneous in nature and evolvable in the laboratory as a bet-hedging strategy under a frequently changing environment (441). Another recent study in yeast evolved under mixed glucose-galactose conditions resulted in the *de novo* emergence of clonal phenotypic heterogeneity resembling a bet-hedging strategy. Here, however, the outcome of populational diversity was likely a response to negative frequency-dependent interactions rather than a bet-hedging strategy (442).

DIVERSITY IN EXPERIMENTAL EVOLUTION

A critical question on the evolution of life on earth is how it became so enormously diverse. The mechanisms that generated diversity at this macroscale, over various ecosystems interconnected by different degrees of migration and evolved over such a long time scale, are clearly very complex. Evolution experiments, however, allow for diversity to be studied under much simpler conditions. Here, diversity can be considered either between replicate populations or within one population and across different levels of biological organization, from phenotype to genotype.

Interpopulational Diversity

Parallel evolution at different levels. Replica lines in experimental evolution often show similar evolutionary responses and, hence, limited interpopulational diversity. A high degree of parallelism is usually found at the level at which natural selection acts, namely, fitness in a given environment (62, 189). In addition, parallel changes are also present across lower levels of the biological organization, like phenotypic traits that might contribute to the change in fitness (66). For example, *M. extorquens* populations all adapted to a newly introduced pathway for growth on methanol in the same phenotypic way, by decreasing the costly overexpression of the exogenous pathway (443) and by changing their entire transcriptome in a synonymous way (97). Also, in the LTEE, gene expression changes were found to be highly similar (224). Similarly, populations evolving resistance to a certain kind of antibiotic often show similar increases in resistance, and even the changes in patterns of antibiotic susceptibility to other, not-selected-for antibiotics have been shown to be highly conserved (85, 114, 130, 131).

Indeed, traits that are only indirectly correlated with a fitness increase can also change in a parallel way between replicate populations (232). The pattern of resistance to different phages, a trait under relaxed selection, changed, for example, similarly in all populations of the LTEE throughout 45,000 generations (444). Pathways and processes underlying the fitness or phenotypic changes also show frequent parallel optimization (110). In the emergence of doxycycline or chloramphenicol resistance, transcription and translation processes are always changed along with membrane transport systems (85). Similarly, mutations or mutated genes in 640 *S. cerevisiae* populations adapting at a large population size showed only a low level of interpopulational parallelism. Grouping the mutated genes by biological functions, however, increased the level of parallelism between the lines and showed clear convergent evolution (259). Even down to the level of operons or genes, parallelism is frequently observed in the sense that genes are often found to be hit multiple times across replicate populations (37, 98, 259, 262, 270), and while this can be the result of mutational hot spots (273), detailed analyses of high-throughput whole-population sequencing time series showed that in the case of the LTEE, this is unlikely (260). In extreme examples, unique target genes seem to exist, which are sometimes hit repeatedly by identical mutations in parallel evolving populations (94, 175, 189, 293). Under selection for trimethoprim resistance, dihydrofolate reductase was the single target (85). Such a high level of parallelism is also known for certain other antibiotics (113, 130).

Explanations for parallelism or diversity. These forms of parallelism are usually signs of adaptive responses and have helped distinguish causal mutations from irrelevant ones. However, moving further away from the direct target of natural selection (e.g., looking at phenotypic traits that are less fitness correlated or at correlated fitness in other environments [111, 153, 445, 446]) and down to lower levels of biological organization, eventually to the DNA base pair level, parallelism decreases, and the observed diversity increases. The conceptual logic is that a similar increase in fitness can be caused by the optimization of different traits via diverse genetic networks or processes, each containing multiple possible target genes that can be changed in an adaptive way at various sites, possibly by different mutations per site (110, 447, 448). An extreme example is that genes, albeit being hit repeatedly, are not often hit twice or more by exactly the same mutations over different replicate populations (132, 237, 249), although exceptions exist (114, 175, 259, 270).

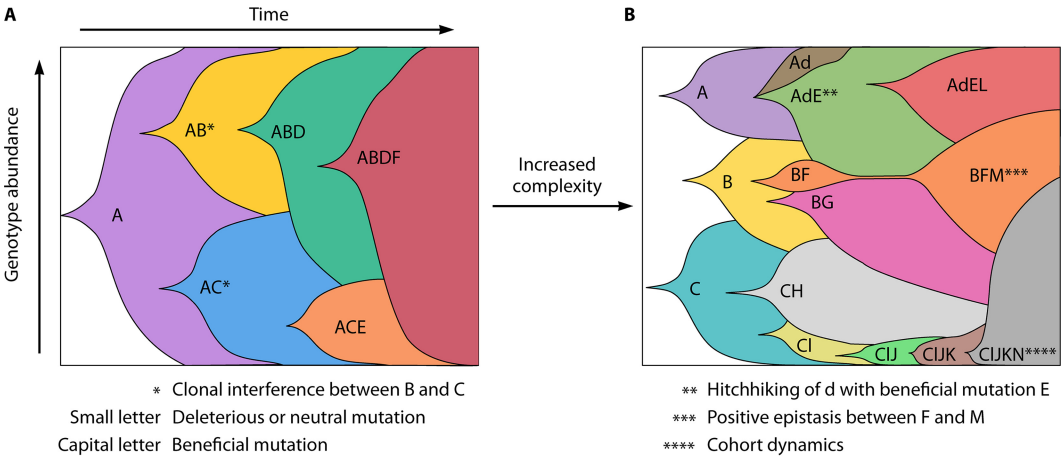

FIG 6 Intrapopulational diversity visualized by Muller plots. The *y* axis shows the relative abundance of a subpopulation's genotype, and the *x* axis shows the evolution of the relative abundance of each genotype over time. (A) In this simple representation of intrapopulational diversity, genotype A occurs and gives rise to two separate subpopulations, AB and AC. Both genotypes B and C are beneficial but cannot be combined due to the absence of sexual reproduction and will clonally interfere. Eventually, beneficial genotype AC will lose from CI with genotype AB successors. (B) Intrapopulation diversity can increase and become more complex, for example, under a higher mutation rate, in larger populations, during evolution on more-rugged fitness landscapes, if there is a large target size for selection to act upon, when an interaction between genotypes occurs, or when evolution proceeds under structured, heterogeneous conditions. CI can then lead to fluctuating selections and soft sweeps, without one genotype purging all others. Here, the rise of deleterious or neutral mutations is also depicted by hitchhiking with beneficial mutations (mutation d in backgrounds A and AE). Some genotypes will show epistasis, e.g., genotype BF, which remains at a low frequency for a long period but rises quickly when genotype M occurs and positively interacts with genotypes F and/or B. Finally, due to epistasis and hitchhiking, cohorts of genotypes arise seemingly simultaneously in an adapting population, such as cohort CIJKN. (Adapted from reference 309.)

Differences between replicate lines could simply reflect differences in speeds or paths taken to climb the same peak in the fitness landscape. Alternatively, populations could indeed be climbing different peaks, leading to more-pronounced and sustained diversity over time. In both cases, diversity between replicate populations will be higher if more beneficial mutations are available (wider peaks and more paths) or if more alternative and mutually exclusive optimization solutions exist (more peaks and a rugged landscape) (74, 98, 448). Diversity should therefore increase if evolution experiments are carried out with smaller replicate populations, which would lead to a larger effect of chance and faster spreading of mutations (less CI), a better exploration of the genotype space in the fitness landscape among replicate populations, and a higher chance of ending up on different peaks (449, 450). Extreme examples are MA experiments where interpopulational diversity increases during the experiment, although natural selection is, of course, very limited, and fitness usually decreases (61, 329). However, the effect of population bottleneck sizes can be more complex, as in the adaptation of *P. fluorescens* toward rifampin resistance, where intense or weak bottlenecking resulted in a higher diversity of genetic resistance mechanisms than in the intermediate situation (451). Similarly, the kind of selection and its strength can influence diversity. Just as some stressful conditions might have more possible adaptive paths than others, increasing selection might decrease the number of accessible solutions and has been shown to limit diversity (132, 143, 148, 191).

Intrapopulational Diversity

Similarly to interpopulational diversity, diversity within a single population clearly depends on the structure of the fitness landscape, the mutation rate, the adaptive mutational target size of selection, and the nature of the selection force. However, at this level, increasing the population size or the mutation rate generally results in higher diversity (Fig. 6). Nevertheless, this diversity will normally be lost by hard sweeps, i.e., *de novo*-generated beneficial genotypes that are fixed by natural selection, thereby replacing the previous ancestor and removing diversity (Fig. 6A).

Clonal interference and soft sweeps. As stated above, CI in large populations can slow down the action of natural selection (see "Clonal interference," above). As such, hard sweeps of beneficial mutants on their way to fixation are slowed down, and simultaneously, the diversity is purged more slowly. In cases with extremely strong CI (e.g., very large populations, high mutation rates, or a large target size for adaptive evolution), selection will not be able to keep up with purging diversity, and hard sweeps will be rare. As a consequence, different adaptive mutations will arise repeatedly in different haplotypes without reaching fixation, also called soft sweeps (Fig. 6B) (452, 453).

The domination of evolutionary dynamics by a succession of soft sweeps, and thus (temporarily) preserved diversity, is frequently seen in the large populations of chemostats that do not experience population bottlenecks like the serial transfer regime does (36, 37, 42, 73, 270). Even during the adaptation of *E. coli* to the mouse gut, a chemostat-like *in vivo* system, high genetic diversity was created by many soft genetic sweeps that, on the phenotypic level, however, resulted in a hard sweep (199). Also, during serial transfer, strong CI can result in high diversity because of soft genetic sweeps (247, 248, 293), which was also observed in the LTEE (231). In extreme cases, in a fluctuating environment with strong trade-offs where conditions change too rapidly to allow for a complete takeover of the population by the current adaptive specialist, ultimate soft sweeps can occur, which act on standing genetic variation and thus cause changes only in the frequencies of specialists that are already present in the population. Standing genetic variation could initially be generated before environmental changes occur by *de novo* mutations. Alternatively, these mutants could already be present in the culture before the start of the evolution experiment. In both cases, this standing genetic variation has been shown to be more important in the beginning of an adaptive trajectory than *de novo* mutations (154, 412, 454, 455). A recent experiment on yeast adapting to antifungals showed that *de novo* mutations, together with standing variation, can influence the future adaptive trajectory but that standing variation clearly imposes a selective threshold for these new arising mutations (456). As such, temporally fluctuating environments have been shown to increase and/or sustain diversity (155, 457, 458), although spatial heterogeneity in the environment is still regarded as the most important factor for diversity to arise (see "Mixed environments with heterogeneity in niches," below).

Sustained diversity from negative frequency-dependent selection. Diversity generated by strong CI, without any form of interaction, will ultimately be lost once the genotype on the highest point of the landscape sweeps to fixation. A more sustained form of diversity in a homogeneous environment arises from negative frequency-dependent selection (NFDS). Here, the fitness of a genotype is inversely correlated with its frequency in the population. Such a pattern predicts some kind of social interaction and helps to explain the high intraspecific diversity in large populations in nature, for example, in bloom-forming plankton (459).

NFDS is present in evolution experiments with communities of multiple interacting species, such as coexisting mutualistic partners or prey and predator. Here, the success of a predator or one of the mutualistic partners is high when their numbers are low compared to the numbers of prey or other partners. Combined with coevolution, in the form of ARD or FSD for the predator-prey system, this can result in a stable coexistence of the interacting partners. In fact, FSD contains another form of NFDS since predator types that target the dominant prey genotype will be selected, and this will result in a higher fitness for alternative prey genotypes that are present at a low frequency (172, 174, 176, 177, 460).

Sustained diversity because of NFDS can also evolve under well-mixed conditions limited by a single carbon source and started with only one founding genotype (73, 461). A classic example under these conditions is diversification into so-called cross-feeders, where one genotype becomes specialized in metabolizing the single carbon source and others adapt to metabolize the products generated by the first. For example, during the adaptation of *E. coli* to glucose limitation in chemostats, genotypes

that benefit from glycerol and acetate excreted from the glucose specialist emerged, which enabled their coexistence by NFDS (46, 462, 463). Similar, strong NFDS based on cross-feeding was found in the Ara-2 population of the LTEE, with the sustained coexistence of two genotypes, L and S (for "large" and "small" based on their colony morphologies), for over 12,000 generations (229, 464). Although this situation is more complex than just simple cross-feeding on excretions and also depends on the death rates in stationary phase and cannibalism of one type on the other (465), the fitness of S or L was shown to be simply inversely proportional to its frequency in the population (466). These cross-feeders can further adapt as interacting but separate sublineages (comparable to the coevolution of two separate species) (467, 468), which causes their stable levels to fluctuate slightly over time. The fact that their community can be disassembled and reassembled because of their cooperative interactions nevertheless shows that genetic diversity is sustained by clonal reinforcement rather than by CI (469). Massive population sequencing in all the lines of the LTEE recently showed that NFDS likely underlies pervasive quasistable, long-term coexistences in many of the populations throughout 60,000 generations (260). While CI likely also explains part of the observed diversity in the LTEE, only NFDS can explain why coexisting clades emerged in which subsequent mutations were fixed.

If selection leads to the loss of costly and leaky but essential functions in part of the population, the Black Queen hypothesis (BQH) (referring to the Queen of Spades, a playing card just like the Red Queen from the RQH, which is to be avoided in the game Hearts) predicts a yet stronger form of coexistence by NFDS (470). While part of the population benefits from the loss of the Black Queen function (the beneficiaries), the remainder of the population is stuck with the function and is obliged to carry it out (the helpers), and since neither them can exclude the other (NFDS, since the function is essential), this leads to some sort of commensalism or mutualism (471) (a distinction between altruists and helpers on the one side and cheaters and beneficiaries on the other, with an overview of many possible Black Queen functions, can be found in reference 472). The BQH was initially used to explain adaptive gene loss in nature resulting in dependence on other cooccurring organisms, as for *Prochlorococcus*, an oxygen-producing photoautotroph in the ocean that lost many mechanisms for detoxifying molecules, leading to oxidative stress, and became dependent on other members in its community (471). Recently, however, the BQH resulted in the stable intraspecies coexistence of diversity in the laboratory. In an environment containing hydrogen peroxide, the founder *E. coli* population split up into two subpopulations: one genotype lost the costly detoxification process, while the other maintained the leaky Black Queen function (473). A similar adaptive loss of a Black Queen function (the assimilation of vitamin B_{12}) was also recently observed to cause a stable coexistence between a beneficiary alga that lost the capacity for vitamin B_{12} production on the one hand and a helper bacterium and the wild-type alga that both rescued B_{12} production on the other (474).

Evolution in Heterogeneous and Structured Environments

The fact that intrapopulational diversity can evolve and be maintained by NFDS under homogeneous conditions indicates that different niches have been created. Indeed, as discussed above, cross-feeders cannot evolve within *E. coli* populations under glucose limitation without the excretions of new, different carbon sources by the glucose specialist. For the evolution of diversity, the presence of multiple niches (heterogeneity) is believed to be an important factor (475). In an attempt to study the emergence of diversity and the coexistence of two or more specialists, a process usually called adaptive radiation, evolution experiments have been carried out in heterogeneous environments containing multiple niches with or without spatial structure (183, 447).

Mixed environments with heterogeneity in niches. While designed as a single-niche-selective environment, the long-term evolutionary process of the LTEE showed that it was in fact carried out in a multiniche environment, eventually leading to the

stable coexistence of multiple specialists (241). In particular, the LTEE environment contains citrate as a potential carbon source besides glucose. The Cit^+ phenotype that evolved is an extreme example of a niche specialist that coexisted with the Cit^- glucose specialist, which actually also evolved cross-feeding behavior on excreted C_4 metabolites of the Cit^+ genotype, in further support of their coexistence (476).

Other experiments intentionally started with an environment with multiple niches to induce diversity (155, 161, 181, 477). A mixture of acetate and glucose resulted, for example, in the emergence and NFDS-based coexistence of two *E. coli* genotypes (180, 447), both of which were specialized either to switch to acetate after glucose depletion or to use glucose (250, 478). The latter simultaneously also excretes acetate, which is believed to have helped in the development of the acetate specialist (178, 479). Apart from different types of nutrients, diversification is also observed in the evolution of *P. fluorescens* simultaneously challenged by different types of predators, resulting in defense specialists (173). Diversification of a unique predator in multiple specialized predators also seems to have happened in a system that started with only one predator and one host after the host diversified under coevolving conditions (171). In the evolution of phage lambda simultaneously presented to a mixture of two hosts, both expressing different receptors for lambda infection routes, the initial generalist rapidly diversified into specialists, even to the extent that the two specialists showed the first signs of speciation (341).

Sometimes, for example, in the combination of glucose with either lactose or maltose or in complex undefined media altogether, evolution did not result in diversification and stable coexistence (155, 181, 182, 194, 477). While this could be due to the limited evolutionary time given in these experiments, another explanation is that the niches are insufficiently distinct from one another (i.e., too few trade-offs exist between specialists), and specialization to either of them does not allow for the avoidance of competition and, thus, the stable coexistence of multiple specialists (181) (see Selection for Specialists or Generalists?, above).

Spatially structured environments. In addition to heterogeneous but well-mixed environments, the presence of structure in the environment is also believed to be important for the evolution of diversity. Structured environments are nowadays frequently used in experimental evolution, and they can induce heterogeneity and thus cause potentially different niches to arise. Seminal experiments in this regard are those on the radiation of *P. fluorescens*. In one such experiment, 3 different morphotypes readily evolved in static broth, each adapted to a different part of the culture (the smooth morph is specialized to the bulk, the fuzzy spreader is specialized to the bottom, and the wrinkly spreader is specialized to the air-liquid interface) and stably coexisted by NFDS (98, 183). Although oxygen availability clearly is an important aspect of each niche, radiation into phenotypes can also be evoked in well-mixed environments where cultures are simultaneously challenged with predators (125), while the presence of predators in static cultures can, in fact, decrease diversity (480). Furthermore, the propensity to radiate is highly dependent on niche position and width of the ancestor. A very confined initial niche, or specialist ancestor, would allow for diversity to arise and endure, while ancestors with less-confined niches that partially overlap the potential new variants have shown less-adaptive radiation behavior (481). Similar adaptive radiation was also observed during the evolution of *B. cenocepacia* in biofilms, structures notorious for their medical importance and known to harbor many different niches (187, 262, 482). In *P. aeruginosa* populations evolving under a regime that optimizes dispersal, attachment, and biofilm formation, diversification was already apparent from the many different colony morphologies (483). One of the causes, increased diversity in the cyclic di-GMP content, enabled variants with high levels, as early colonists, to make way for increased biofilm production by the entire population. In the evolution of vesicular stomatitis virus, while the simultaneous presence of different hosts selected for generalists rather than multiple specialists, diversity in generalists within populations was also increased in the heterogeneous structured environments (484).

Even one of the simplest setups possible that still includes structure, the growth of populations on agar plates with no clear niches at the start, resulted in more-diverse populations than in the situation under mixed-liquid conditions. Another experiment using *P. fluorescens* showed that in simple expanding colonies, selection for increased territorial expansion already occurs. Within 10 days, *de novo* social behavior develops by the emergence and coexistence of a genotype that pushes the lubricant-producing ancestor forward (257). Additionally, populations on a plate can also be regarded as many small populations that evolved semi-independently in parallel, which can also lead to more diversity (43, 184). A more dynamic form of diversity in a rock-paper-scissors-like game among three *E. coli* strains, a toxin producer, a toxin-resistant mutant, and a mutant that was neither a producer nor resistant (see "Nontransitive fitness and the Penrose staircase," above), could be maintained only if played in a structured environment where boundaries to diffusion and migration exist (390). Likewise, the commensal interaction between *Acinetobacter* excreting benzoate when growing on benzyl alcohol and *Pseudomonas putida* metabolizing this benzoate benefits from a structured environment in the form of a biofilm (176). Another cooperative behavior between *S. enterica* serovar Typhimurium and *E. coli*, where the former evolves increased methionine excretion to serve the latter, from which it receives carbon sources, could emerge only under a structured environment of a plate where the benefits of the cooperation are predominantly received by cooperators (485). In these structured circumstances, positive frequency-dependent selection (PFDS) can promote and sustain diversity. As such, natural isolates of *M. xanthus* showed increased fitness when abundant, which acts as a form of territorial behavior thwarting invasion but promoting diversity when genotypes vary patchily in structured environments (486). In the same species, structured environments were recently also shown to increase diversity between populations adapting to an environment selective for the increased expansion of swarming colonies (487). To study adaptation of large populations, microbial evolution and growth arena (MEGA) plates were designed (488). The MEGA plate incorporates, in addition to spatial structure, a sense of directionality, as selective pressure is varied not in time but in a spatial sequence (Fig. 7). When applying this method to adaptation to antibiotic pressure, resistance increased rapidly, by 5 orders of magnitude. More importantly, Kishony's group visualized and sampled the adaptive routes and their massive diversity that evolved over time and in space (488). By doing so, they also showed that the fitness of a mutant is a complex trait that cannot simply be reduced to the level of resistance but actually can also depend on the time and place of its emergence and its capacity to be mobile, etc. As a consequence, some highly resistant mutants became trapped in space behind more-sensitive lines and thereby did not contribute to the population's ultimate evolutionary path, an effect that likely occurs under many natural conditions as well (Fig. 7).

Although structure in the environment clearly often promotes diversity, exceptions exist (448). For example, for the evolution of sustained diversity by cross-feeding in *E. coli*, structure has been shown to have negative impacts, probably because of reduced diffusion of metabolites (489). Likewise, if a multispecies community consists of only a toxin producer and a sensitive genotype, structure promotes invasion and take-over by the toxin producer, even at very low initial frequencies (490). Clearly, the specific kinds of ecological mechanisms and interactions maintaining diversity can impact the effect that environmental structure has.

CONCLUSIONS AND FUTURE PERSPECTIVES

Experimental evolution with microorganisms has clearly deepened our understanding of the dynamics of evolution, such as the consequences of asexual reproduction, the prevalence and importance of interactions between single mutations, and how natural selection can select suboptimality. Furthermore, it has helped in investigating several evolutionary theories, like the ones that predict the evolution of either specialization or generalism, depending on trade-offs and changes in the environment, or the ones that describe how diversity can be maintained by combinations of CI and soft

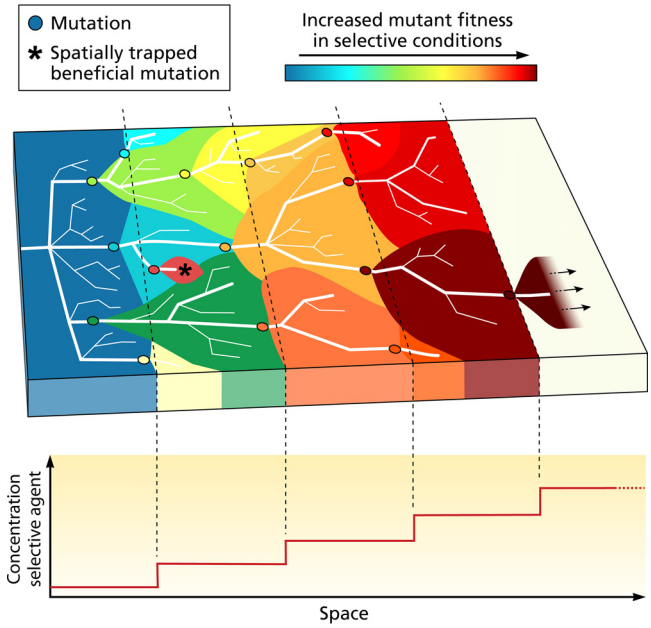

FIG 7 Evolution in a spatially structured environment. Shown is a MEGA (microbial evolution and growth arena) plate setup as a prime example of evolution in a spatially structured environment (488). The plate consists of adjacent regions containing stepwise increased concentrations of a selective agent. The wild-type strain is inoculated at the left side and rapidly grows in the region with the lowest concentration of the selective agent. Growth stops at the intersection with the region containing a higher concentration of the selective agent. The subsequent occurrence of different adaptive mutations allows growth in this region until it reaches the next boundary. This setup enables visualization of evolution in action, demonstrates the power of mutations for adaptation under stressful conditions, and allows for the isolation and determination of individual mutants that arise. This setup also shows the impact of a spatially structured environment on the outcome of an evolution experiment. The red mutant (marked with *) becomes spatially trapped due to fast growth of the light blue and dark green mutants. (Based on data from reference 488.)

sweeps, NFDS, and evolution on rugged landscapes in heterogeneous and/or structured environments. In addition, experimental evolution has become a well-established and powerful research tool to improve strains for biotechnological applications or to help understand the mechanisms behind, or the evolution of, more-specific physiological traits, like antibiotic resistance, multicellularity, or kin discrimination. The future use of experimental evolution will clearly benefit from more-clever or more-complex setups, longer experiments, and more inclusion of communities in experimental designs. As such, evolution experiments can allow for a more detailed examination of the interplay between ecology and evolution or of important evolutionary transformations, like the transition from single-cell life to multicellularity and the concurrent division of labor, the emergence and importance of sexual reproduction, and the appearance of new species. In addition, technological improvements, especially in the three following fields, will likely spur on the further maturation of evolution experiments and their widespread implementation.

First, NGS technologies will continue to improve. Existing technologies will have an increased output/cost ratio and improved accuracy and read length and will enable a higher order of multiplexing in their library preparation at lower prices. As a result, the analysis of the genomic dynamics during evolution can be carried out in greater detail and parallelism and will be available to many more laboratories. Following the example of Levy et al. (248), massive lineage tracking could reveal the DFE of beneficial mutations in many other species, across many replicate populations under different selective pressures, and could allow the examination of the change of its shape throughout evolution. In addition, newer technologies, such as nanopore technology (491, 492) or single-molecule, real-time (SMRT) sequencing (493), will become more readily accessible, although the first reports of their use in experimental evolution have

appeared already (351). Based on different technologies, they provide extremely long reads of DNA (to enhance *de novo* sequencing, the detection of larger rearrangements, or the identification of mutations in repetitive DNA) and detect DNA modifications (494–497), and as such, they could facilitate, for example, the exploration of epigenetic evolution.

Experimental evolution will also benefit from the increased use of laboratory automation, as this will expand the throughput of experimental evolution while limiting human errors, without an excessive increase in labor demands. Especially, the introduction of microfluidic systems to the field of experimental evolution will result in great benefits because of their miniaturization, further automation, and gains in throughput and potential for more-detailed analyses, such as those available through microscopic imaging (77, 498–500). The increased throughput provides more statistical power to experimental evolution to make general statements (77), as in a recent report on the generality of diminishing-returns epistasis between adaptive mutations (259).

Third and finally is the progress made in the field of genomic engineering. Until now, most reports have used more-classic tools, if any at all, to change a species' genome. These tools, however, often suffer from low effectiveness and efficiency, making the construction of many (combinations of) mutations time-consuming. Some tools allow for genome engineering on a wider scale, with greater ease, and in a more diverse set of species (63, 501–504); recently developed CRISPR-based tools are especially promising (350, 505). In addition, for species capable of efficient sexual reproduction, crossing evolved and ancestral genotypes and analysis of a constructed bulk-segregant pool can allow one to pinpoint with high throughput the effects of many (combinations of) single mutations identified in evolution experiments (299). The widespread integration of these advanced techniques will, for example, enhance the construction of real fitness landscapes, larger than the ones obtained so far, which were limited to the combination of only a few mutations, and allow a detailed examination of the genetic basis of complex, experimentally evolved traits.

Advances in all these areas are transforming the field of experimental evolution using microorganisms. From a somewhat labor-intensive science useful for testing limited evolutionary theories and exploring interesting changes in the evolution of traits, it has become a powerful, flexible, and unbiased tool that fully harnesses the potential of natural selection, leading to a deeper understanding of evolutionary processes and how microbes function in the light of evolution. Experimental evolution not only allows advanced evolutionary research questions to be looked into but also can be widely used on different scales in a variety of laboratories studying or improving complex microbial traits.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/MMBR.00008-18.

SUPPLEMENTAL FILE 1, XLSX file, 0.4 MB.

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Bram Van den Bergh has a master's degree in Applied Biosciences and Engineering from KU Leuven, Belgium (July 2011). In January 2016, he completed his Ph.D. research funded by the Research Fund Flanders (FWO) on using experimental evolution to study bacterial persistence in the group of Jan Michiels at KU Leuven. During his FWO-funded postdoctoral research, he is further exploiting this approach in various ways, both *in vitro* and *in situ* in animal models. Currently, he is an EMBO long-term fellow at Cornell University, funded by BAEF and FWO, while maintaining his affiliation with KU Leuven and the Flemish Institute for Biotechnology (VIB).

Toon Swings graduated as a master in Bioscience Engineering in 2012 and as a Ph.D. in Bioscience Engineering in 2017 from KU Leuven, Belgium. Currently, he is working as a postdoctoral scientist at the VIB and the Centre of Microbial and Plant Genetics in the Jan Michiels group. His work focuses on evolutionary mechanisms leading to higher ethanol tolerance in *Escherichia coli*, the role of hypermutation, and new CRISPR/Cas9 applications for genome engineering.

Maarten Fauvart graduated as a master in Applied Biosciences and Engineering, specialization Cell and Gene Biotechnology, in 2003 and obtained a Ph.D. in Bioscience Engineering in 2008. During his time as a postdoctoral fellow at the Centre of Microbial and Plant Genetics, KU Leuven, he cosupervised 10 Ph.D. students and published over 50 papers in international, peer-reviewed journals. Currently, he is working as R&D Team Leader Life Science Technologies at imec, a world-leading research and innovation hub in nanoelectronics, nanobiology, and digital technologies.

Jan Michiels is full professor at the KU Leuven Faculty of Bioscience Engineering. He is head of the Symbiotic and Pathogenic Interactions group (SPI) and of the Centre of Microbial and Plant Genetics (CMPG). His research group is furthermore part of the VIB. He teaches courses on molecular biology, genetics, and bacterial physiology. His research focuses on molecular aspects of microbe-host interactions and stress resistance in bacteria, using experimental evolution in particular as a powerful tool. He has published over 100 articles in international journals with peer review.