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REVIEWS

Review

Experimental Evolution as a Tool to Investigate Natural Processes and Molecular Functions

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The experimental evolution (EE) of microbes has allowed evolutionary biologists to examine adaptive processes in real time, generating novel insights into fundamental laws of evolution. Less appreciated is the potential of this approach to advance our understanding of microbial cells and molecular processes as a complement to traditional molecular genetics. The tracking of mutations underlying phenotypic changes offers the opportunity for detailed molecular analyses of novel phenotypes. This provides a breadth of information on diverse biological systems and may retrace key past events of natural history. Here, we highlight how the field has advanced our understanding of gene regulation, antibiotic resistance, and host–microbiome interactions to exemplify how EE can be used to provide new light on microbial systems.

Experimental Evolution: Beyond Evolutionary Biology

Artificial breeding of plants and animals had a key role not only in the maturation of Darwin's theory of evolution by natural selection, but also in the rise of modern human societies (from agriculture to recreational breeding of plants and pets). Nonprofessional evolutionary biologists performing (sometimes unwittingly) evolution experiments have successfully improved the yield of many biological processes. By contrast, although some evolutionary biologists contemporary of Darwin turned to **experimental evolution (EE;** see Glossary), the influence of this approach on the development of evolutionary biology has only come to prominence over the past few decades [1]. Applied to microbes with short generation times in various conditions (Box 1), EE has generated unique biological material (Figure 1) that was used to probe the evolutionary dynamics of microbial phenotypes [2,3], to decipher the genetic bases of adaptation [4], and to optimize microbial traits for industrial use [5].

Despite these successes, many biologists remain unaware or unconvinced of the relevance of EE approaches to their research programs. Typical criticisms of EE include the artificial nature of the experimental setups or their simplicity in terms of biotic and abiotic interactions [6]. However, this is also true for many excellent lines of research in molecular biology, which depend on the construction of simplified and well-controlled setups. Here, we argue that EE has matured into a rich field with a varied set of tools that can help microbiologists to unravel molecular processes underlying adaptive phenotypes. This is independent of the relevance of EE to reproduce natural adaptive processes, since laboratory phenotypes can be interesting on their own. For example, improving growth rate, yield, and recombinant protein production in the laboratory has obvious biotechnological interest [7]. Contemporary microbes can also be used to experiment on the ecological conditions and evolutionary patterns that might have accompanied the evolution of multicellularity millions of years ago [8]. Here, instead of providing an exhaustive overview of the field (for which we refer the interested reader to recent reviews [2–4,7–12]), we use a few selected examples of gene regulation, antibiotic resistance, and host–microbiome interactions to illustrate how EE contributes to our understanding of fundamental aspects of molecular biology and

Highlights

EE can complement traditional molecular genetic studies on microbial systems.

The diversity of EE approaches has resulted in significant progress in many fields of microbiology, including molecular mechanisms of gene regulation, antibiotic resistance, and host–microbiome interactions.

Under specific conditions, EE can parallel the evolution of natural systems.

EE offers exciting perspectives to discover the function of new genes and probe evolution within communities.

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helps in the manipulation of natural ecosystems. Given that the natural inclination of most biologists is to understand if the phenotypes observed in laboratory-evolved mutants are relevant to understand natural processes, we present both examples of discrepancies (Box 2) and of meaningful parallels (Box 3) between EE and natural evolutionary processes. While this review focuses on bacteria, concepts discussed here are also relevant to other organisms, especially viruses or unicellular eukaryotes.

Gene Regulation

A major challenge for modern biology has been to map and understand the logics of gene regulatory networks (GRNs). Outstanding enigmas in molecular biology tackled by EE include how the

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Box 1. The Many Faces of Experimental Evolution.

EE can be performed under a range of experimental settings adapted to the biological question of interest. Details on the design of these studies are reviewed elsewhere [3,90]. In an attempt to classify the most common EE practices, one can distinguish levels of complexity along two criteria: environmental conditions and starting biological material (Figure I).

Starting Biological Material
Wild-type bacterial strains are commonly used to start evolution experiments. Although in theory any cultivable strain can be used, most studies focus on fast-growing genetically tractable model bacteria. Other studies use modify-and-evolve approaches where genetic engineering is used to delete [32], introduce a trait [23,27,75], or modify a gene or the whole genome [91,92], and is followed by EE to understand how the system evolves. These approaches profit enormously from the recent developments in synthetic biology and CRISPR-based technologies. Comparative evolution studies are typically performed by evolving several independent lines from the same ancestor in parallel, although can also involve different strains and/or species exposed to the same conditions [93]. Complexity in starting material can be increased by putting together different bacterial species [94,95] or using complex communities [96]. In these cases, experimentalists can follow either the evolution of one of the organisms or the coevolution of multiple organisms.

Environmental Complexity
Following the trends set by the long-term evolution experiment (LTEE), most microbial evolution experiments are performed in simple growth conditions, such as in shaken Erlenmeyer flasks [97] or in chemostats [98], with a variety of volumes, time delay, and volume of transfer between subsequent cycles (flasks) or dilution rates (chemostats). Several studies used more complex environments. Spatially structured environments are generated in static liquid cultures (creating an oxygen gradient [99]), on solid supports within liquid medium [100], or on solid surfaces (agar plates [25]). In this case, microorganisms deplete resources locally, leading to differences between patches. Growth on solid media or in liquid metapopulations also helps to manipulate genetic assortment between neighboring cells, a condition often used to explore the evolution of social behaviors [101–103]. The strength of selective pressures can be adjusted during the experiment by varying antibiotic concentrations in a continuous culture [58], creating chemical gradients in agar plates [104], or by varying conditions between successive growth cycles [20,43]. Finally, some studies mimic natural conditions more closely by using more complex settings, such as eukaryotic hosts, to study the evolution of mutualists or pathogens [66,77,81,105–107].
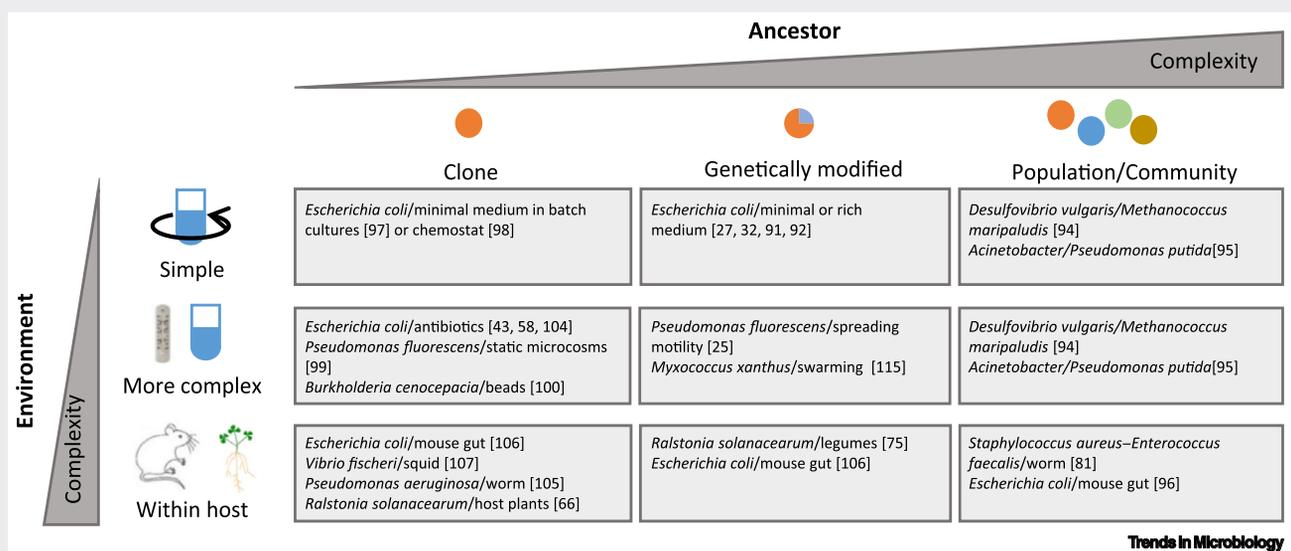

	Clone	Genetically modified	Population/Community
Simple	*Escherichia coli*/minimal medium in batch cultures [97] or chemostat [98]	*Escherichia coli*/minimal or rich medium [27, 32, 91, 92]	*Desulfovibrio vulgaris*/*Methanococcus maripaludis* [94] *Acinetobacter*/*Pseudomonas putida*[95]
More complex	*Escherichia coli*/antibiotics [43, 58, 104] *Pseudomonas fluorescens*/static microcosms [99] *Burkholderia cenocepacia*/beads [100]	*Pseudomonas fluorescens*/spreading motility [25] *Myxococcus xanthus*/swarming [115]	*Desulfovibrio vulgaris*/*Methanococcus maripaludis* [94] *Acinetobacter*/*Pseudomonas putida*[95]
Within host	*Escherichia coli*/mouse gut [106] *Vibrio fischeri*/squid [107] *Pseudomonas aeruginosa*/worm [105] *Ralstonia solanacearum*/host plants [66]	*Ralstonia solanacearum*/legumes [75] *Escherichia coli*/mouse gut [106]	*Staphylococcus aureus–Enterococcus faecalis*/worm [81] *Escherichia coli*/mouse gut [96]

Trends in Microbiology

Figure I. Experimental Settings in Experimental Evolution (EE). Examples of experiments using biological material or environments exhibiting increasing levels of complexity [17,19,24,37,50,57,66,72,84–92,96–99,107].
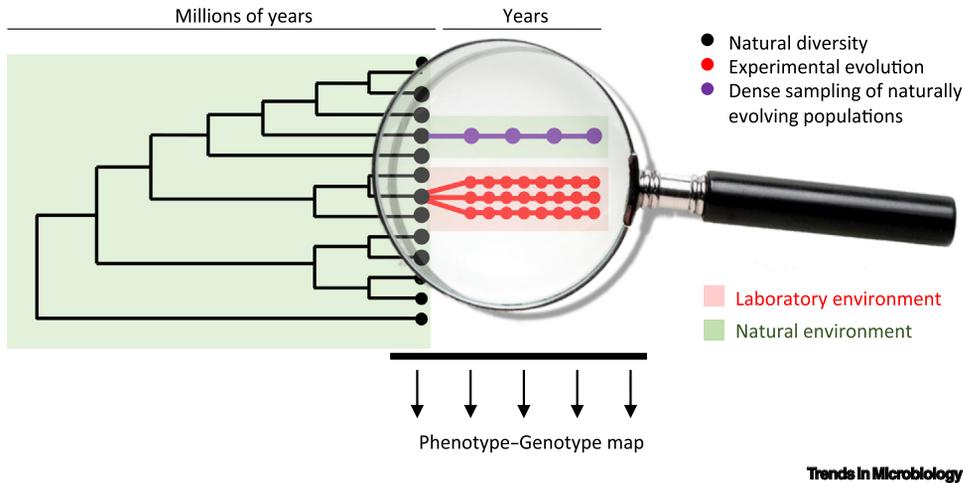
Figure 1. Experimental Evolution (EE): A Source of Biological Material Available for Phenotypic and Genotypic Analysis. Traditionally, only extant or recent populations resulting from millions of years of natural evolution are available for analysis. EE allows the analysis of all steps of adaptation during years of accelerated evolution in controlled conditions, thanks to frozen fossil records. Recently, the intensive genomic sampling of variants in natural populations provided data that can be compared with that of EE. Points represent available naturally or experimentally evolved bacterial clones and/or populations.

interactions of transcription factors (TFs) with DNA arise and evolve, why they are so hard to identify correctly, and how they form complex networks [13].

The cooption or modulation of bacterial regulatory responses provides a multitude of opportunities for adaptation to new environmental challenges. Indeed, most adaptive mutations in EE are found in regulatory regions or regulatory genes [4]. Detailed molecular analyses of the associated transcriptional rewiring can reveal new components from signaling networks, new connections between known regulatory components, and new crosstalk between signaling and metabolic pathways. These trends emerged from some of the very early studies in experimental evolution [14,15]. A prominent example includes the evolution of a new lactose fermentation system in

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Box 2. Divergences between EE and Natural Evolution.

Many EE studies identified patterns of molecular evolution with large excesses of nonsynonymous adaptive mutations, whereas natural populations systematically show a predominance of synonymous substitutions caused by purifying selection on protein sequences [108]. This may result from the joint effects of the simple continuous unidirectional selective pressures applied in many EE together with the use of conditions free from most other constraints that bacteria endure in natural environments. The same reasons may explain why core genes tend to accumulate few substitutions in natural populations, but evolve faster in the LTEE [109]. The contrast between evolutionary patterns is particularly striking for the RNA polymerase gene *rpoB*, which often accumulates adaptive mutations in *in vitro* EE experiments but is extremely conserved in natural evolution [4]. Interestingly, a recent *Escherichia coli* EE study in the mouse gut, a more natural environment, showed lower rates of evolution and no mutations in *rpoB* [110]. Similar discrepancies were found by a study of *Pseudomonas aeruginosa* adaptation to the airways of patients with cystic fibrosis in which over 200 000 generations resulted in limited genetic diversification. In contrast to *in vitro* EE, the *in vivo* process revealed an initial period of adaptive mutations followed by a period with the more usual pattern of dominance of purifying selection [111]. Another reason for the excess of nonsynonymous adaptive mutations in EE is the lack of sexual exchanges with distant strains or species in most EE setups. This prevents the income of adaptive changes by HGF and results in adaptive mutations touching key processes that are highly conserved in nature. For example, many traits are lost in EE because they are costly in simplified setups, but they are under selection and, thus, conserved in nature [112]. A striking example of this contrast is given by the frequent evolution of mutators during phage–bacteria coevolution in simple experiments, which was not observed in complex environments closer to natural conditions [113,114].

Box 3. Parallels between EE and Natural Evolution.

The use of more complex setups in EE has found interesting parallels with analogous processes in natural history. For example, diversification of *Myxococcus xanthus* during EE led to genetic diversity close to that identified in natural populations sampled from small parcels in the soil [115]. EE of *Burkholderia cenocepacia* in biofilms revealed a wealth of mutations associated with its adaptation and diversification, of which four broad classes were also found in clinical isolates of *Burkholderia dolosa* and *Pseudomonas aeruginosa* from patients with cystic fibrosis, suggesting a parallelism between adaptation to the biofilm lifestyle and lung colonization [100]. A recent study aimed to compare directly the patterns of evolution of resistance to colistin in *P. aeruginosa* using both laboratory EE and the analysis of four clinical isolates from a single patient with cystic fibrosis (sampled within a period of 3 months) [60]. This revealed a complex, multistep adaptation process requiring epistatic mutations in several loci where parallels between the natural and experimental processes could be identified: all resistant mutants were mutators and the evolution of resistance occurred through mutations in *prmB*, part of the PrmAB two-component system. This showed that processes at comparable timescales requiring a relatively straightforward adaptation process can reveal significant parallels. Another study combining detailed phenotypic characterization and mathematical modeling showed that high mutational supply, influenced by population and bottleneck sizes, was a key parameter favoring parallelism between laboratory and natural evolution of ciprofloxacin resistance in *Escherichia coli* [116]. More complex adaptation processes were investigated by evolving three clones of *Lactococcus lactis* from a plant isolate to the dairy niche [117]. Gene expression differences between the parental and the dairy strain were maximal at an operon encoding an ABC transporter that was expressed 350 times more in the dairy strain. Interestingly, this operon was also expressed at a higher level in two of the three adapted strains. An even more radical EE, leading to a change in lifestyle from a plant pathogen to a rhizobial mutualist upon acquisition of a large plasmid carrying the symbiosis genes and after a few hundreds of generations (see the main text), also showed striking parallels to the natural process that took place more than 10 million years ago [118]. Despite the radically different time span of the evolutionary processes and degree of achievement of the symbioses, adaptation was accompanied in both cases by an overall pattern of purifying selection. Both natural and experimental processes showed little sign of adaptation in the fast-evolving symbiotic plasmid, whereas many adaptive mutations took place in the genetic background of the bacteria, including mutations that led to the co-option of the same quorum-sensing system in both processes. These studies show that EE reveals significant parallels to natural history when it mimics key conditions of the natural processes.

Escherichia coli, via mutations in the *egbA* β-galactosidase and its regulator [16]. More recently, EE has uncovered a novel **small (s)RNA** regulating *Myxococcus xanthus* **fruiting body** development. *M. xanthus* responds to nutrient starvation by generating fruiting bodies containing stress-resistant spores. This behavior was lost during EE in nutrient-rich liquid medium, but was subsequently re-evolved in alternating cycles of starvation and nonstarvation [17]. The point mutation responsible for the re-evolution of fruiting-body formation was found in a previously unannotated intergenic region, leading to the discovery of the novel sRNA controlling this developmental pathway [18]. Another study established a link between pyrimidine metabolism and the Gac/Rsm signaling pathway, a major determinant of life-style switch in *Pseudomonas* spp. [19] that controls the production of extracellular capsules in *Pseudomonas fluorescens*. Evolution of *P. fluorescens* under fluctuating environments gave rise to a strain that produces a subpopulation of capsulated cells [20]. This phenotype is underpinned by a mutation that decreases pyrimidine biosynthesis [21] and increases ribosome production [22]. That both increased ribosome levels and functional Gac/Rsm signaling are required for heterogeneous capsule production shows that central metabolism (pyrimidine biosynthesis) can alter the output of a two-component signaling system [22].

The plasticity of regulatory regions and TFs observed in EE can be recruited to understand fundamental properties of transcriptional control. Low-affinity TF–DNA interactions appear to have a critical role in the evolution of GRNs. Yona *et al*. [23] replaced the *lac* promoter with random DNA sequences of the same size in *E. coli* and observed that bacterial growth in the presence of lactose rapidly led to the evolution of functional promoters. A single mutation in synthetic promoters was sufficient to induce substantial expression in most cases (Figure 2A). The rapid *de novo* evolution of promoters and the **functional promiscuity** of TFs may explain the intriguing **pervasive transcription** observed in bacterial genomes [24]. It may also explain the evolutionary plasticity of gene expression in bacteria, since an initially weak binding of a TF on a regulatory
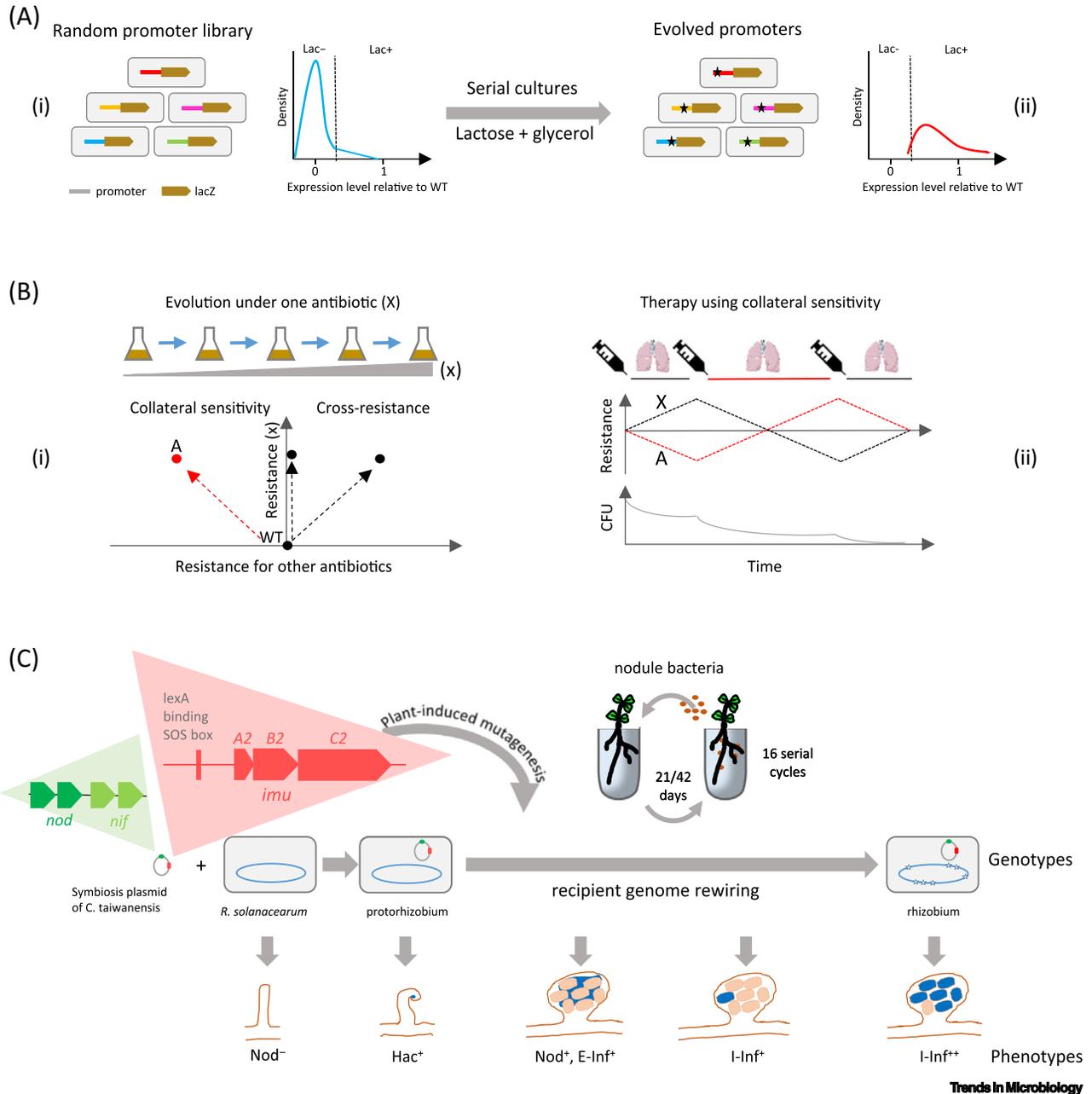
Figure 2. Selected Evolution Experiments That Have Contributed to Advances in Gene Regulation (A), Antibiotic Resistance (B), and Host–Microbe Interactions (C). (A) Evolution of promoters [23]. (i) The *lac* promoter was replaced by random sequences in *Escherichia coli*. Bacteria were evolved by serial dilutions in 0.05% glycerol (utilized by the strain) and 0.2% lactose (originally not utilized). (ii) Following laboratory selection, ~60% of promoters had acquired a single mutation (black star) and exhibited on average 50% of the wild-type (WT) activity. (B) Evolution of collateral sensitivity and its use to design new therapeutic strategies [44]. (i) Bacteria selected to resist a given antibiotic (X) reproducibly display an increased (cross-resistance), unchanged, or decreased (collateral sensitivity) resistance to other antibiotics (e.g., A) compared with their WT ancestor. (ii) Patterns of collateral sensitivity can be exploited by cycling antibiotic treatments that accelerate the eradication of bacterial pathogens. (C) Evolution of new legume symbionts [77,78]. The symbiosis plasmid of *Cupriavidus taiwanensis* was introduced into *Ralstonia solanacearum*, generating a nonnodulating protorhizobium (Nod⁻) that was further evolved using serial cycles of co-culture with *Mimosa pudica*, the natural host of *C. taiwanensis*. The symbiosis plasmid has the essential *nod* and *nif/fix* genes required for nodulation and nitrogen fixation. In addition, it contains *imuA2B2C2* genes encoding stress-responsive error-prone DNA polymerases that transiently elevated the mutation rate of bacteria growing in the rhizosphere. In only 16 cycles

(Figure legend continued at the bottom of the next page.)

region can be reinforced by mutations in the DNA-binding domain. This was observed during the re-evolution of motility in *P. fluorescens* following the deletion of the master regulator of flagellar synthesis *fleQ* [25]. Under selection for motility, flagellin expression was restored within 96 h in a two-step process involving: (i) the increased phosphorylation (and, thus, activation level) of a nitrogen-related transcriptional regulator with weak (promiscuous) activity on flagellar genes; and (ii) a switch-of-function mutation that redirected transcriptional activity of this regulator from nitrogen uptake to flagellar genes. The plasticity of regulatory interactions can also be associated with **noise in gene expression**, which has attracted interest in recent years because it can contribute to phenotypic variability in clonal populations [26]. Wolf *et al.* [27] showed that libraries of random promoters evolved to produce GFP at high or intermediate expression levels had lower average noise than natural *E. coli* promoters. This suggests that noise is a selected trait in nature. When the average production of a protein is not optimal in a given environment, a broad (i.e., noisy) distribution of gene expression levels increases the likelihood that some cells of the population express an appropriate amount of this protein. Mathematical modeling suggests that noise can act as a primitive form of gene regulation and pave the way towards more precise regulation [27]. Altogether, these studies illustrate that what may appear to be 'nonoptimality' in transcriptional regulation (functional promiscuity and noise) in fact drives the evolution of GRNs.

EE enables us to directly probe systems-level properties of GRNs in an evolutionary context. Witnessing how GRNs are gradually built by evolutionary tinkering (rather than by optimized design) is key to understanding their complex architecture and emergent properties, such as robustness, resilience, or evolvability [28]. In many cases, transcriptional changes induced by genetic or environmental perturbations are reverted during the early steps of adaptation by adaptive mutations in global regulators that restore cellular homeostasis [29–32]. These results support the idea that transcriptional stability is essential for optimal fitness and can be restored by minimal genetic modifications in global regulators, thus highlighting the resilience of GRNs. However, the topology of GRN architecture can also benefit bacterial evolvability. Studying the evolution of the **bow-tie network** controlling flagellar production in *E. coli*, Ni *et al.* [33] found that motility in a porous environment (the trait under selection in their experiment) can increase via mutations in multiple target genes; yet, all adaptive mutations commonly modified the activity of a core sigma-factor checkpoint controlling flagellar gene expression. By allowing multiple mutations in independent genes to target the same central regulator of motility, the bow-tie architecture provides evolutionary flexibility for the fine-tuning of bacterial behaviors. These examples show that EE is a powerful tool to analyze evolutionary properties of GRNs.

Antibiotic Resistance

The emergence of antibiotic-resistant bacteria is a remarkable example of human-induced evolutionary process. Resistant bacteria emerge systematically within a few years of the introduction of every novel antibiotic. The consequences are dramatic: 700 000 deaths per year, estimated to increase to over 10 million in the incoming decades[i]. EE is particularly well suited to decipher the mechanisms of acquisition of antibiotic resistance given the timescale and intensity of selection in this process. This has produced unexpected novel lines of research. For example, early studies on the EE of antibiotic resistance kick-started the study of microbial mutagenesis by identifying the first *E. coli* **mutators** [34]. The simplicity of tracking resistant bacteria, the societal relevance of the topic, and its implications for molecular genetics and physiology have stimulated

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(~ 400 generations) the ancestral protorhizobium, which was only able to induce root hair curling (Hac[+]) allowing the formation of infection sites, successively acquired the capacity to enter the root and form nodules (Nod[+]), extracellularly infect nodules (E-Inf[+]), intracellularly invade nodules (I-Inf[+]), and massively invade nodule cells (I-Inf[++]), via genome remodeling. Stars symbolize mutations. Bacteria are represented in blue in nodules. Adapted from [74,78].

research on the mechanisms of acquisition of resistance, on multiple resistance, and on the compensation of the fitness costs of resistance.

Most resistances studied from clinical isolates are strong, because of the obvious medical interest of such cases. EE provides complementary information about resistance in controlled setups where pathways to resistance can be tracked, selection forces tuned, and physiological states controlled for. Notably, EE revealed the important role of low (below the minimum inhibitory concentration; MIC) antibiotic concentrations in the evolution of resistance. These concentrations can be encountered in many types of environment and do not result in cell death, but provide sufficient pressure for the selection of resistant variants and even the evolution of novel mechanisms of resistance [35]. They also accelerate the acquisition of resistance by selecting for mutators [36] and by increasing the rates of horizontal gene transfer (HGT) [37]. Lindsey *et al.* [38] evolved hundreds of populations of *E. coli* under variable rates of increase in the concentration of rifampicin. By assessing the fitness of genetically engineered combinations of mutations from isolates evolved under low rates of environmental change, they showed that certain genotypes were evolutionarily inaccessible to evolution under rapid environmental changes. Wistrand-Yuen *et al.* [39] then showed that *Salmonella enterica* exposed to **sub-MIC levels** of streptomycin evolved high-level resistance via mechanisms different from those observed under above-MIC conditions. Sub-MIC resistance evolved through small-effect mutations that combined to confer high-level resistance.

Antibiotics stimulate a set of core responses in bacterial physiology [40], which contributes to explain why resistance to one antibiotic can change the susceptibility of the cell to others [41]. Several recent studies detailed the network of **cross-resistance** and **collateral susceptibility** resulting from EE of resistance to each of a large range of antibiotics [42–44]. Evolution involving cross-resistance and collateral sensitivity is frequently convergent in *E. coli*, meaning that one can predict, to a reasonable extent, the antibiotic-resistance phenotypes from the genome sequences of the laboratory-evolved lines [45]. Thus, treatments based on alternating drugs with compatible collateral sensitivity profiles could be more efficient and lead to the slower development of resistance (Figure 2B) [44]. Several of these studies observed that populations adapted to resist to aminoglycosides show systematically lower fitness in the presence of other types of antibiotics. In molecular terms, this could be the consequence of selection for alterations in the inner membrane potential reducing the uptake of aminoglycoside-related antibiotics, which would simultaneously lower the activity of efflux pumps using proton-motive force [42]. Interestingly, the comparison of interaction networks evolving in conditions of weak and high antibiotic concentration revealed that the strength of selection shapes the acquisition of resistance: cross-resistance tends to be stronger under higher antibiotic concentrations [46]. The stochasticity of evolutionary trajectories leading to resistance to one antibiotic can influence the occurrence of collateral sensitivity [47], calling for a careful assessment of the robustness of this phenomenon when wanting to exploit it in a clinical setting. Together with the studies on the effect of sub-MIC conditions, this suggests that the path towards resistance, involving exposure to high or low antibiotic concentrations, will shape the network of collateral effects of antibiotics.

The many evolution experiments detailing the mutational landscape of antibiotic resistance have systematically revealed rapid partial compensatory evolution of the initial fitness costs of resistance [48–50]. Compensation facilitates the spread and fixation of antibiotic-resistant lineages. For example, rifampicin resistance in *Mycobacterium tuberculosis* is costly in laboratory-derived mutants, but multidrug-resistant clinical strains often show no fitness defects [51] because of compensatory mutations [52]. The study of mutants arising in *E. coli* in the presence of fluoroquinolones showed that compensation of fitness cost associated with antibiotic resistance

can lead to bacteria that are as fit as the wild-type susceptible bacteria in the absence of antibiotics [53]. Prolonged colonization of chickens with fluoroquinolone-resistant *Campylobacter jejuni*, a somewhat less controlled evolution experiment, revealed that resistant bacteria were also fitter pathogens [54]. Genes that simultaneously increase virulence and resistance may in fact be common. They have been observed in several other pathogens, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Vibrio cholerae* [55]. Even costly resistance mutations can coexist with other genotypes for hundreds of generations when their high adaptive potential counteracts their initial cost [56]. This means that a multistep process of the slow accumulation of mutations conferring resistance and cost compensation, as expected under sub-MIC conditions, can result in bacteria that are both resistant and fit. This process is strongly dependent on the genetic context of the mutations (epistasis) [57–60]. For example, a comparison of streptomycin and rifampicin double-resistant *E. coli* with single-resistant clones obtained through EE showed that low-fitness double-resistant bacteria compensate their cost faster than single-resistant strains because of the acquisition of compensatory mutations with larger effects [61]. Surprisingly, some mutations only compensate for double resistance, being neutral or deleterious in single-resistant backgrounds. This means that multiple resistances may not rapidly go away with pauses in the use of the corresponding antibiotics.

Host–Microbe Interactions

Plants and animals are persistently inhabited by microbes, the contribution of which to host health, nutrition, and development is increasingly recognized [62,63]. Elucidating the functional mechanisms and evolutionary potential of host–microbiome interactions is crucial to manipulate this ecosystem and improve host health.

Most bacterial pathogens have the remarkable ability to alternate between external environments and specialized host niches. Rapid and coordinated shifts in metabolism, physiology, and virulence factor production in response to environmental changes are orchestrated by multilayered and highly complex circuitries that are difficult to decipher [64]. Adaptation to new hosts, which is generally rapid in laboratory conditions [65], has the potential to reveal new components of virulence pathways. This was recently highlighted in an evolution experiment aiming at adapting the plant pathogen *Ralstonia solanacearum* to different host plants [66]. Beneficial mutations improving *in planta* colonization mainly occurred in one gene, which was named *efpR* (for enhanced fitness in plants). The *efpR* gene was shown to encode a transcriptional regulator acting as both a central player of the *R. solanacearum* virulence network and a global catabolic repressor downregulating the expression of multiple metabolic pathways [67]. Experimental adaptation of *R. solanacearum* to a non-host legume further identified other components of the *efpR* pathway [68]. Although the genetic bases of virulence in *R. solanacearum* had been amply dissected [69], this pathway had not been previously identified, illustrating how EE coupled with genome resequencing allows the identification of novel molecular players of biological functions.

Many ecological transitions towards pathogenic or mutualistic symbiosis include an initial acquisition by HGT of mobile genetic elements (MGE) that can provide complex novel traits in a single event of transfer [70,71]. Profound changes in lifestyle may require the remodeling of the metabolic and signaling networks in the recipient genome, a process that may take hundreds to millions of years *in natura* [72]. The study of this process by the analysis of extant microorganisms can be complemented by EE to pinpoint specific molecular mechanisms that ensure the full expression of acquired traits. This is illustrated by the study of nitrogen-fixing legume symbionts (rhizobia), which evolved through the acquisition of a set of essential symbiotic genes [73]. An experiment was designed to evolve a plant pathogenic bacterium (*R. solanacearum*) into a legume

symbiont under plant (*Mimosa*) selection pressure (Figure 2C). This experiment showed that control mechanisms developed by the host progressively shaped the bacteria via the multistep selection of compatible bacterial traits [74]. The gradual activation and improvement of the first symbiotic properties (i.e., the induction of root nodules where bacteria fix nitrogen and the infection of these nodules) occurred via the inactivation of the *R. solanacearum* pathogenic type III secretion system and regulatory rewiring [75–77], demonstrating the requirement of post-HGT modifications to achieve symbiosis in the case of horizontal transfer between distantly related bacteria. Upon experimentally replaying rhizobia evolution, a genetic mechanism was discovered to accelerate symbiotic evolution [78]. Evolved *R. solanacearum* underwent a transient hypermutagenesis stage that occurred at every inoculation cycle before the cells entered the plant. Investigating the role of *imuABC* error-prone DNA polymerases present on the transferred symbiotic plasmid provided evidence that this mutagenesis cassette is expressed in stress conditions (outside the host plant), thus increasing genetic diversity and offering more phenotypic diversity for plant selection. *imuABC* cassettes were found on ~50% of the symbiotic plasmids, supporting the hypothesis that this hypermutagenesis mechanism has facilitated the evolution of new rhizobia *in natura*. After 400 generations, mutualistic nitrogen fixation was not achieved, possibly because time was too short. Yet another evolution experiment performed with the natural symbiont of *Mimosa* (*Cupriavidus taiwanensis*) showed that, because of host sanctions occurring at the postinfection level, rare nitrogen-fixing symbionts (that may arise via mutation during evolution) progressively invaded a population dominated by nonfixing bacteria, with a probability that depended on ecological factors [79]. This provided a better understanding of the spread of the mutualistic trait during natural evolution [73].

Plant and animal microbiomes comprise complex and dynamic bacterial consortia, the interspecific interactions of which have implications for the host [80]. The importance of bacterial antagonism for the evolution of infection was demonstrated in a tripartite interaction between *Caenorhabditis elegans* and two bacterial pathogens. In a first EE, King *et al.* [81] showed that mildly pathogenic bacteria (*Enterococcus faecalis*) living in worms rapidly evolved increased competitiveness against a more virulent pathogen (*Staphylococcus aureus*). This reduced the mortality caused by *S. aureus* infections. The mechanistic basis for protection was an increased production by *E. faecalis* of antimicrobial reactive oxygen species directly affecting pathogen growth. Although this broad-spectrum defense mechanism was not novel, it showed that microbes living within a host can become mutualists in response to infection by other pathogens. A subsequent EE showed that microbe–microbe interactions within hosts can drive the evolution of pathogens. To limit *E. faecalis* colonization, which exploits the costly siderophores of *S. aureus*, the latter evolved to produce fewer siderophores [82]. Since siderophore production contributes to virulence by improving pathogen growth in iron-limited hosts, its diminution leads to less-virulent clones. Hence, bacterial antagonistic interactions can modulate the production of virulence factors and, consequently, influence how the microbiome impacts the host.

Concluding Remarks and Future Perspectives

EE has already provided a wealth of information on the molecular events (see [83] for additional examples) and conditions driving phenotypic adaptation in several model biological systems. We expect that many more discoveries will follow. For practical purposes, characterization of adaptive mutations has usually focused on known genes and pathways. A large number of adaptive mutations found in genes of unknown function remain to be analyzed, representing a challenging untapped reservoir of new discoveries. The development of cross-experiment databases (such as the recent ALEdb [84]) may help identify promising candidate genes to initiate such studies. Moreover, the introduction of novel non-model bacteria allows EE to tackle different biological questions, such as studying the evolution and functioning of microbial communities

Outstanding Questions

Can we exploit mutations found in EE to characterize genes of unknown function?

Can EE reveal mechanisms slowing down the evolution of resistance?

Can the EE of microbial communities bring new insights into their functional characteristics?

Can we use within-host EE to identify host factors (and other environmental factors) shaping bacterial evolutionary trajectories during infection?

Can EE reproduce the emergence of major intracellular symbiotic associations (such as mitochondria in eukaryotic cells, plastids in the green lineage, association between fungi and land plants, or obligate endosymbionts of insects)?

(see Outstanding Questions). EE can also provide an alternative to genetic screens for organisms that are not genetically amenable.

EE can be used to study mechanisms of evolution, and their underlying molecular biology, independently of the events that actually took place in the natural history of the species. Yet, it would be of interest to use EE as a tool to test hypotheses about evolution in nature, especially in cases where the study of natural populations only provides limited insights into ancient processes. EE is not necessarily relevant in this context because of its simplified setups, and numerous reports have revealed differences between EE and natural processes (Box 2). Nevertheless, there is a growing number of reported parallels between natural and EE, especially in studies where the latter tried to match more closely the conditions of natural evolution (Box 3). The extent to which EE studies can mirror the natural evolution of bacterial traits is likely to be a fertile area for future research.

On the technological side, recent genome-editing technologies, including CRISPR-Cas or 'deep mutational scanning', accelerate the exploration of genotype–phenotype landscapes [10]. Moreover, new cultivation procedures (particularly those based on micro/millifluidics [85,86]) will allow large-scale, automated evolution experiments, and their genetic analysis can be facilitated by DNA barcoding [10]. Therefore, with continuously decreasing sequencing costs, the EE field is ripe for appropriation by molecular microbiologists combined with imaginative selective regimes, original microbial strains or communities, and novel biological questions. More generally, the adoption of EE by a wider community of microbiologists could accelerate the march towards the much-needed synthesis of molecular and evolutionary approaches [87–89].

Resources

[i]https://amr-review.org/Publications

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