New insights into bacterial adaptation through *in vivo* and *in silico* experimental evolution

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Abstract | Microbiology research has recently undergone major developments that have led to great progress towards obtaining an integrated view of microbial cell function. Microbial genetics, high-throughput technologies and systems biology have all provided an improved understanding of the structure and function of bacterial genomes and cellular networks. However, integrated evolutionary perspectives are needed to relate the dynamics of adaptive changes to the phenotypic and genotypic landscapes of living organisms. Here, we review evolution experiments, carried out both *in vivo* with microorganisms and *in silico* with artificial organisms, that have provided insights into bacterial adaptation and emphasize the potential of bacterial regulatory networks to evolve.

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The most salient feature of life has been the stability of its bacterial mode from the beginning of the fossil record until today and, with little doubt, into all future time so long as the earth endures. This is truly the "age of bacteria" — as it was in the beginning, is now and ever shall be.[1]

Bacteria have lived on our planet for billions of years, during which time they have adapted to many fluctuating and harsh environments and have colonized virtually all available ecological niches. Today, with the help of more than one million referenced scientific studies, covering all of biology as well as physics, chemistry, mathematics and computer science, we are beginning to understand how billions of years of evolution have shaped these complex organisms.

Bacterial genetics has generated a wealth of information through analyses of mutant strains that were selected to elucidate important pathways in microbial physiology, biochemistry, metabolism and gene regulation. More than 1,500 microbial genomes have been sequenced, including hundreds of *Escherichia coli* isolates, revealing a high level of recombination and genetic diversity. The development of tools to analyse global expression patterns and metabolic networks, such as transcriptomics, proteomics, metabolomics and fluxomics, provided great insights into the organization of cellular networks. Moreover, the combination of fluorescent reporters and microscopic and microfluidic technologies allowed a change in the scale of analyses from the population to the individual-cell level. These developments have favoured the integration of new modelling approaches from the fields of mathematics, physics and computer science.

Bacteria, and especially *E. coli*, have been the workhorses for much of modern biology, including systems biology, the multidisciplinary approach to investigating organismal complexity. However, ~40% of bacterial genes still have unknown functions. Most genetic studies rely on selective conditions in the laboratory without appreciating the pace of natural selection. Systems biology generally focuses on reference clones and provides a static description of networks within a species. Comparisons between distantly related species can give an overview of network variability but provide little insight into network dynamics and its underlying causes and constraints. However, even on short timescales, bacteria are not static. Evolution is one of the major ways in which they deal with drastic environmental variations. Bacteria are structured by evolution, and their structures influence their further evolution. However, not all structures are equally likely to appear by random mutation, be fixed by selection or drift and change rapidly enough to cope with environmental variations. A full understanding of bacterial cells therefore needs to include both an evolutionary framework and an integrated approach if we are to relate genomic and regulatory changes to phenotypic properties such as fitness and evolvability. Ultimately, such a fully integrated evolutionary framework should provide general principles for microbial

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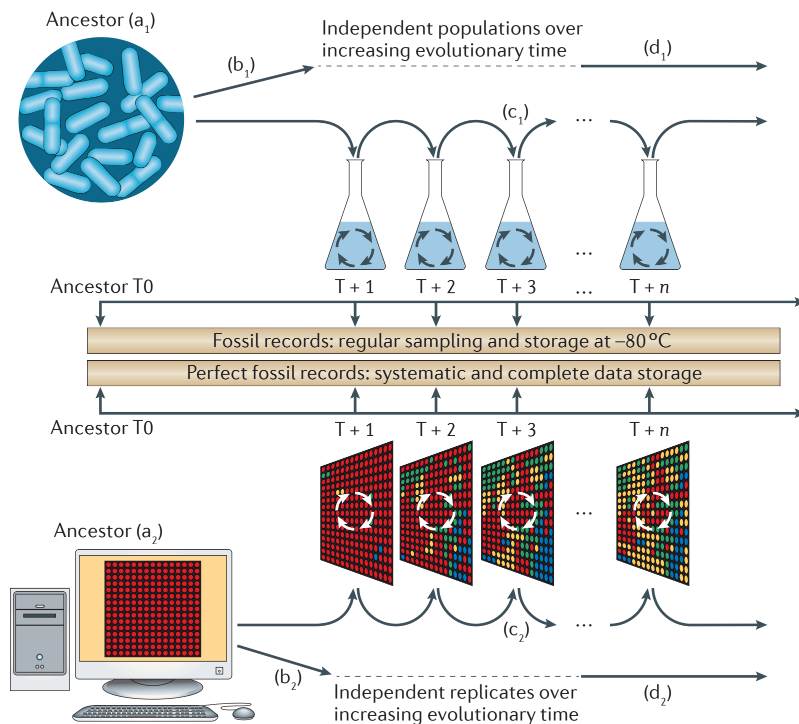

Figure 1 | ***In vivo* microbial and *in silico* evolution experiments.** Ancestral microbial organisms (top) or artificial organisms (bottom) are propagated in defined wet or computer environments, respectively. The main advantage in these experiments is the availability of an ancestor and the evolved populations that are sampled throughout evolution. All living and artificial organisms can be frozen or stored in databases, respectively, and revived at any time for further analyses. Many parameters can be varied. *In vivo*, the ancestor (a_1) can be any microorganism, the only constraint being its ease of cultivation; ancestral digital organisms (a_2) can be randomly constructed, or designed to have capabilities such as replication or minimal metabolism. The number of replicate cultures (b_1 and b_2) can be varied, leading to independent populations derived from the common ancestor. *In vivo*, culture conditions can be varied, including the media, the physical parameters, the structure of the environment (batch or chemostat culture; heterogeneous or homogeneous environments), the effective population size and the bottlenecks at each transfer (c_1). *In silico*, almost all parameters can be tested independently or in combination, including mutation rates, mutation biases and selection strength, which define the way in which the *in silico* transfer (c_2) is carried out. The total duration (d_1) and sampling times of *in vivo* experiments can be varied; *in silico* experiments classically run for hundreds of thousands of generations (d_2).

Variability
The potential or propensity of the phenotype to vary (whether or not it actually does in the present population or sample). This depends on the rates and patterns of mutation and recombination, and on the genotype–phenotype map.

Fitness
An integrated measure of the relative survival and reproductive rate of genotypes in a given environment.

Evolvability
The potential or propensity of the phenotype to vary in a possibly adaptive manner.

adaptation, as well as global laws that link evolutionary processes and organismal structure. However, using such an evolutionary perspective is difficult, mainly because the relevant events that resulted in the present organismal structure occurred at some unknown time in the past, in unknown conditions and with unknown constraints. Thus, specific tools are necessary to directly observe evolutionary dynamics and relate these events to the real conditions in which they occur.

The past three decades have seen the emergence of experiments that are designed to reproduce evolution in controlled conditions in the laboratory[2] and, more recently, on a computer[3]. Laboratory *in vivo* evolution experiments focus on the single most important, integrative and complex phenotype of all: fitness. They allow rigorous connections to be made between genetic changes and phenotypic outcomes in a complex adaptive system, such as a bacterial cell. The adaptive mutations that are discovered during evolution experiments are often subtle in their phenotypic effects and therefore different from those observed in more traditional genetic studies, in which genes are typically knocked out and selective screens usually rely on extreme 'plus or minus' phenotypes. In parallel — and often independently — evolution experiments have also been conducted on artificial, non-living substrates. For two decades, computer simulations and *in silico* experimental evolution approaches have been developed, in which artificial organisms (so-called digital organisms) evolve in a computational environment. In these digital experiments, practitioners are aware of all possible evolutionary events (including variations that appear and are not further selected for), and the experiments are highly reproducible, and can be carried out in multiple contexts and under multiple evolutionary conditions. *In vivo* experimental evolution enables a better understanding of the pace of evolution and its main features in living organisms[2,4]. When combined with molecular biology and high-throughput technologies, it also allows phenotypic variations to be related to the molecular events that occurred in the course of the experiment[5–7]. *In silico* experimental evolution can bypass species-specific traits and generate more general observations.

Here, we review *in vivo* and *in silico* evolution experiments for bacteria; although there have also been reports of these experiments for viruses[8,9] and higher eukaryotes such as *Drosophila melanogaster*[10], they are not discussed in this Review. We focus especially on new insights from experimental evolution that link global microbial phenotypes (such as physiology and behaviour) with molecular and regulatory observations. We also discuss the limits of experimental evolution, as well as future perspectives, including the need for closer collaboration between researchers using *in vivo* and *in silico* approaches.

In vivo and *in silico* experimental evolution

Biological systems emerge through Darwinian evolution, which is characterized by random genetic modifications followed by selection of well-adapted individuals[11]. This combination of 'chance and necessity' can be studied efficiently by propagating organisms in controlled environments (FIG. 1). This strategy, called experimental evolution, provides complementary advantages to most classical genetic studies (BOX 1). Owing to their short replication times, large populations and easy storage[2], microorganisms are excellent candidates for use in experimental evolution[4,6,7] (TABLE 1). Replicate populations have been propagated from microbial ancestors over different evolutionary timescales, from tens to tens of thousands of generations, and under diverse environmental conditions. These different environments impose selection for changes in either specific phenotypes (including growth in the presence of inducible or non-native substrates, and resistance to stresses such as antibiotics, atypical pH or temperature) or broad phenotypes (such as growth in the presence of preferred carbon sources[12–17] or fluctuating levels of resources[18]; social behaviours, including differentiation and the production

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Box 1 | **Advantages of evolution experiments over classical genetic studies**

In vivo microbial evolution experiments provide complementary and additional power to classical genetic studies, for the reasons listed below.

- Most genetic studies are based on stringent selective media, only allowing the growth of mutants with, for example, large gain-of-function mutations[148]. These classical genetic studies often rely on gene inactivation or on modification of residues that strongly affect protein function, and thereby on phenotypic screens with 'plus or minus' effects. By contrast, evolution experiments select for mutations with more subtle effects, providing opportunities for identifying new functions or functional domains within genes and proteins.
- Evolution experiments do not rely on prior knowledge about gene function and are exclusively based on the ability of cells to evolve and adapt under selective conditions.
- Evolution experiments provide a time frame during which direct comparisons can be performed between an organism and its descendants. All ancestral and evolved clones have isogenic backgrounds. Phenotypic and genetic changes are therefore amenable to rigorous analyses, both dynamically and quantitatively. Independent lineages are available to assess the reproducibility of evolutionary pathways.
- *In silico* evolution experiments are complementary tools that can help decipher the mechanisms identified *in vivo;* they do this by constructing alternative scenarios or null hypotheses that would often constitute impossible *in vivo* experiments[149]. Although simulations are limited by the simplifications that are inherent to any model, they allow perfectly controlled conditions, parameter exploration, multiple repetitions and exhaustive records of all mutations and lineages, including those that go extinct during the experiment.

of public goods[19–25]; bacterium–plant interactions[26]; ploidy; sex; and speciation[27]). Some evolution experiments have also been initiated using mutant strains as ancestors, with the mutations affecting either a specific trait (such as the synthesis of a particular enzyme) or a more global one (such as the synthesis of a DNA repair enzyme, a global regulator or a key component of central metabolism) to analyse the mutational process[28,29] and the robustness[30] and adaptability of biological networks[31–35].

Evolution experiments have also recently been designed with virtual 'organisms' that reproduce and mutate. From an algorithmic viewpoint, *in silico* experimental evolution, or "digital genetics" (REF. 3), is very close to evolutionary computation, in which Darwinian evolution is used to solve engineering problems[36]. In these *in silico* experiments, however, the evolutionary process itself is studied from an artificial-life perspective in order to unravel some of the general properties of living systems and, notably, of evolution. Indeed, as John Maynard-Smith stated in 1992: "We badly need a comparative biology. So far, we have been able to study only one evolving system… If we want to discover generalizations about evolving systems, we will have to look at artificial ones." (REF. 37.) *In silico* experimental evolution follows similar strategies to the *in vivo* experiments, albeit on digital organisms (FIG. 1). Artificial organisms that are simulated in a computer are submitted to variation and selection processes, yielding a minimal model of Darwinian evolution in which the emergence of particular properties can be studied (FIG. 2). The artificial organisms possess a genetic material that is interpreted by dedicated programs to compute the phenotype. These programs implement a simplified artificial chemistry, allowing the organism to achieve tasks such as resource or input processing. Each individual organism has a reproduction rate that is based on the fulfilment of these tasks in a given 'environment' (modelled by the available resources and by the tasks that enable reproduction) and can undergo various types of mutation during its replication. Hence, a population of digital organisms evolves and adapts to its environment. The precise implementation of variation and selection depends on how the genetic material is encoded and whether resource competition is explicit or implicit. Following the pioneering Tierra system, in which self-replicating computer programs compete for reproduction inside a virtual computer[38], various frameworks have been developed (TABLE 2), each of which contains simplifications in order to focus on the evolution of specific structures and properties.

The similarity of *in vivo* and *in silico* experimental evolution is also obvious in the ways that the results of the experiments are analysed. In both cases, organisms are collected throughout the evolutionary process (FIG. 1) and compared at different levels, from the most global (fitness) to the most local (sequence), depending on the available tools and on the aim of the experiment. The pace of evolution at all levels can therefore be estimated and compared in the two types of experiment to ultimately decipher the complex relationship between phenotypic and molecular dynamics.

Digital organisms
Computational data structures that process resources, reproduce, mutate and therefore evolve. Such 'organisms' are used as tools to study Darwinian evolution.

Robustness
A measure of the invariance of a phenotype in the face of mutational or environmental perturbations. The mechanisms underlying robustness are diverse, ranging from thermodynamic stability at the RNA and protein levels to behaviour at the organismal level.

Ecology and phenotypic innovations

Fitness and phenotypic traits are the first levels available for analysis and therefore the main levels observed by the pioneers of experimental evolution[39–43]. Indeed, evolution experiments provide a quantitative estimate of fitness changes and of the pace of adaptation[12,15,23,26]. Moreover, fitness changes are always associated with major phenotypic modifications, including cell size[44], metabolic reactions[33], global gene expression levels[16,45–47], stress resistance[48–53], production of biofilm-like structures[21,54], sugar uptake[15] and cooperative traits[19,23,25].

The evolutionary dynamics of the phenotypic changes observed in experimental evolution strategies have been extensively reviewed[2,4,6,7], and three major trends can be observed. First, major phenotypic innovations can emerge, including the ability to colonize ecological niches that are not used by the ancestor[23,55]; new growth, metabolic and resistance abilities[39,41,49,50,52,53,56–58]; and the mobilization of new or unused cellular pathways[22,24,31–34]. These innovations emphasize the rewiring potential of biological networks (see below). Moreover, this trend is often observed in the early evolutionary history of populations, although there are some noticeable exceptions[59]. Phenotypic innovations also depend on complex relationships between genomic changes and ecological conditions, as demonstrated recently in the case of an evolved phage λ, which was able to recognize new receptors on *E. coli* cells[60].

Second, in almost all experiments, a high level of phenotypic parallelism[61] has been observed, with repeated similar changes in independently evolved populations[12,15–17,19,23,44–46,50,53,59,62–67]. Identification of the underlying mutations addresses the redundancy, plasticity and

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Table 1 | **General features of microbial evolution experiments**

Organism	Ancestor	Environment	Selective condition	Generations*	Evolved phenotypic changes‡
Escherichia coli	Reference and laboratory strains	Batch culture	Minimal glucose medium	>53,000	Cell size[44], global gene expression, regulatory networks[5] and adaptive diversification[65,72,75]
			Temperature	2,000	Resistance to stress[57,58,62,63]
			pH	2,000	Resistance to stress[49]
			Starvation	150 days	Resistance to stress[48]
			Ionizing radiation	<100	Resistance to stress[50]
			Various carbon sources	1,000	Divergence[18] and global gene expression[45]
			Non-native carbon source	700	New abilities for carbon source utilization[56]
			Mixed carbon sources	1,000	Adaptive diversification[14,72]
		Chemostat	Glucose- or phosphate-limited medium	100–200	Global gene expression, and regulatory networks[15,107]
			Glucose-limited medium	1,750	Adaptive diversification[65]
	Mutant strain	Batch culture	Minimal glucose medium	20 to several hundred	Alternative pathways[31–34]
Salmonella enterica subsp. *enterica* serovar Typhimurium	Laboratory and mutant strains	Solid medium	Rich medium	1,500–7,000	Accumulation of mutations[28,29]
Pseudomonas fluorescens	Reference strain	Static-broth microcosm	Rich medium	≤20 days	Adaptive diversification, cooperation and cheater traits[23]
	Mutant strain	Static-broth microcosm	Rich medium	5 days	Alternative regulatory pathways[24]
Pseudomonas aeruginosa	Reference strain	Static broth	Iron-limited medium	42	Cooperation and cheater traits[25]
Myxococcus xanthus	Reference strain	Agitated batch culture	Rich medium	1,000	Social versus asocial behaviour[20,22]
	Mutant strain	Soft solid medium	Rich medium	64 weeks	Social versus asocial behaviour[21]
Ralstonia solanacearum	Mutant strain	Plant seedlings	Nitrogen-free medium	<25	Bacterium–host interactions[26]
Saccharomyces cerevisiae	Reference strain	Chemostat	Glucose-limited medium	500	Metabolic and regulatory pathways[17,27]
			Glucose-, sulphate- and phosphate-limited medium	200	Metabolic and regulatory pathways[16,27]
	Mutant strain	Chemostat	Amino acid-limited medium and a sugar switch	~10–20	Global gene expression[35]

*The duration of each evolution experiment is given by the number of generations when known, or by another appropriate time unit when not. ‡The list of phenotypic changes that evolved in the different evolution experiments is not exhaustive and includes only major traits. Fitness changes are not included.

interconnectedness of genetic pathways. Indeed, a high degree of genetic parallelism has been detected in association with the phenotypic parallelism[5,15,19,53,62,64,65,68], with the same genes repeatedly being targets of natural selection. This trend was recently confirmed in an evolution experiment in which more than 100 replicate populations were propagated from an *E. coli* ancestor for 2,000 generations at high temperature[62]. However, this is not always true[59] and, even when genetic parallelism is observed, high allelic divergence can be detected in independently evolving populations[62,64,68]. Moreover, different evolved alleles from the same gene can have different phenotypic and fitness effects in the same environment, revealing complex relationships between phenotype and genotype.

Parallelism
The independent evolution of similar traits in replicate lineages that are propagated in similar environments.

Third, complex population structures emerge. For instance, a high level of within-population diversity is observed in all evolution experiments, irrespective of their duration, environmental conditions and ancestor[48,69–71]. This can lead to the emergence of stable polymorphisms[23,65,72,73] even when it is not expected[74], such as in homogeneous environments[75–77]. Moreover, in many cases, evolutionary shifts have been observed that organize single cells into cooperating groups of cells[19,78], with selection acting on the synthesis of public goods[25,54,79–81] and on social traits[20–22,82,83].

To summarize, *in vivo* evolution experiments have revealed how selection allows for the fast adaptation of microbial cells to diverse environments, resulting

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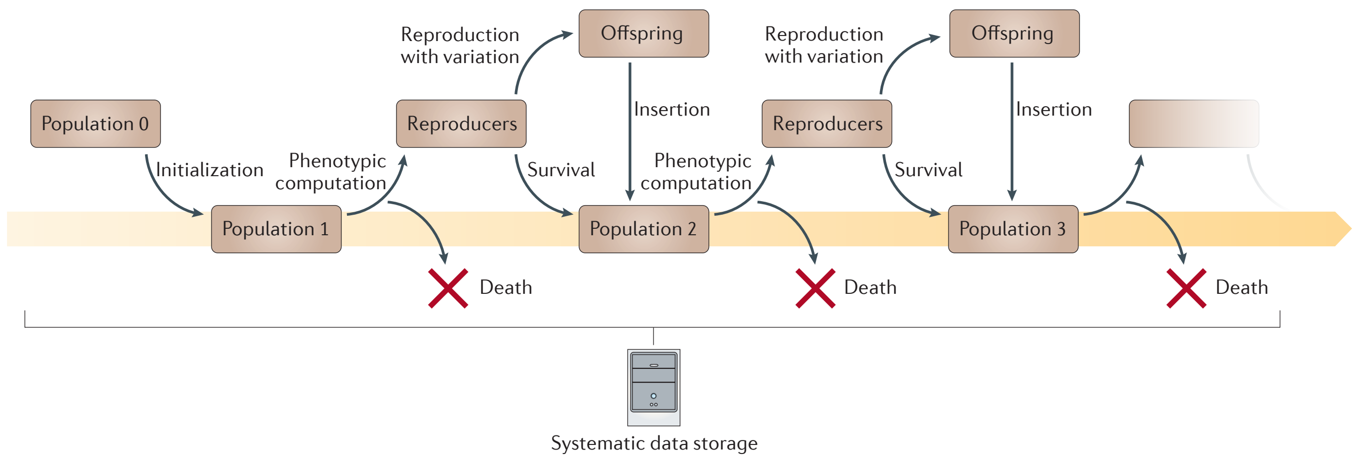

Figure 2 | **Principle of *in silico* experimental evolution.** In digital genetics, the organisms are modelled by data structures, using various formalisms (TABLE 2). Whatever the formalism, the general principles are always the same. During an experiment, a pool of organisms (population 0) is created; organisms may be created randomly — naive organisms — or by hand, depending on the chosen formalism. Experiments can also be initiated with organisms that were issued from a previous, possibly long-term, *in silico* evolution experiment. Evolution is then modelled by a generation cycle: at each generation, the organisms of population n are evaluated, generally on the basis of their ability to perform some predefined tasks; the fittest organisms have more chance of reproducing than the others, so are selected; and finally, the fittest organisms are replicated, with errors. During the replication, a new pool of organisms (population $n+1$) is created and can enter a new cycle. All the organisms at each generation can be stored in databases for subsequent analysis. The generation cycle implicitly generates an evolutionary process that can be studied on its own by analysing the fate of different organisms under different conditions (population size, mutation rates, environmental variation, and so on), for example.

not only in phenotypic improvement and innovation but also in diversification and cooperation. These early observations already question evolutionary theory. Indeed, models predict that diversity patterns are transient in homogeneous environments owing to periodic selection of fitter mutants and niche exclusion[74]. Following the seminal work of Thomas Ray, who observed complex ecological relationships in Tierra[38], *in silico* studies have been used to systematically characterize the conditions required for these specific evolutionary outcomes. For example, adaptive radiation of digital species in spatially homogeneous environments has been associated with the inflow rate of resources[84], and the evolution of cross-feeding (an example of niche construction) has been linked to the strength of selection[85]. Allelic formalisms have been used to investigate the influence of recombination on bacterial speciation[86], and experiments have been performed using the 'program' formalism (TABLE 2) (with the Avida framework) in order to study the pace of evolution and its ability to evolve complex phenotypes[87]. Regarding the evolution of cooperation, digital organisms have been successfully used to test the theoretical prediction that natural selection will favour the evolution of more and more targeted altruism[88]. Similarly, evolving robots were used to quantitatively test Hamilton's rule for the evolution of altruism[89].

Genetic targets of natural selection

Identification of the adaptive mutations that underlie the phenotypic changes in replicate evolving populations provides opportunities to study the modulation of phenotypic traits and its underlying molecular and biochemical mechanisms, to compare the mutational pathways that sustain adaptive evolution and to rigorously analyse the genotype–phenotype map[90] as adaptation occurs. Many genetic tools have been applied to evolution experiments to identify the mutations that account for phenotypic changes[15,21,44,48–51,54,91].

Fitness-enhancing mutations affect either individual genes (or operons) encoding specific metabolic and structural enzymes or regulatory genes ranging from local controllers of gene expression to the most global regulators. Early evolution experiments, in which bacteria carrying mutations in various pathways were propagated under conditions selecting for new metabolic abilities, revealed two types of genetic change that are required for new phenotypes to evolve; the first type of change affects genes encoding structural enzymes, improving their kinetic parameters, and the second type targets local regulatory genes, resulting in constitutive synthesis of the required enzymes[39,40,43]. More recently, following the advent of whole-genome sequencing technologies, similar evolution experiments have been performed using mutant strains with impaired metabolism as ancestors[31,33,34]. The identification of all the mutations that were fixed during evolution confirmed the trends revealed by the earlier experiments, but extended them by showing that other targets of natural selection are genes encoding global regulators that are deeply involved in cellular networks.

In certain experiments, *E. coli* cells were adapted to specific carbon sources such as glucose, glycerol or 1,2-propanediol in batch or chemostat conditions. These experiments revealed mutations in genes that are involved specifically in the transport and/or consumption of these substrates[15,56,92] or in genes encoding

Niche exclusion
The idea that a single niche can sustain only a single genotype.

Niche construction
Environmental changes that are generated by the evolving organisms themselves.

Hamilton's rule
The theory that altruism can be selected for when $rb-c>0$ (in which c is the fitness cost to the altruist, b is the fitness benefit to the beneficiary and r is the genetic relatedness of the two organisms).

Genotype–phenotype map
A representation of how the genetic architecture of an organism produces its phenotype through developmental interactions with the environment.

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Table 2 | **Genome formalisms in *in silico* experimental evolution**

Formalism*	Description	Questions addressed
Program	The genome is a sequence of instructions in a programing language. The fitness of the program depends on its ability to create copies of itself in the computer's memory and/or to perform specific computations	• The emergence of parasites and hyperparasites[38] • The evolution of robustness, evolvability, complexity and modularity[3,87,142,147,150] • The adaptive radiation of species[84] • The information threshold (the maximum amount of information that can be evolutionarily maintained)[151]
Allelic	The genome is made up of a fixed gene number, *n*; each gene can exist in a finite or infinite number of alleles; alleles are represented by integers or characters, and each individual is characterized by its *n* alleles	• The evolution of mutators[135,136] • Bacterial speciation in neutral conditions[86]
Network	The individuals are characterized by a graph representing a gene-regulatory network, a neural network or even a logic circuit; there is no explicit DNA level, and mutations directly change the connections or the node numbers in the network	• The evolution of network evolvability and modularity[122,123,152,153] • The importance of post-transcriptional regulation[124] • The relationship of robustness to mutations and to noise[154] • The evolution of communication, cooperation and altruism[89,155]
String-of-pearls	The genome is a variable-length string of 'pearls' of different types: phenotype genes, transcription factor genes, repeats, retrotransposons, binding sites, and so on; each pearl type can exist in a predefined number of variants; gene number, order and regulation can evolve through mutations and rearrangements	• Genome and network evolvability[141,143] • Resource processing in ecosystems[85] • Sympatric speciation[156]
Sequence-of-nucleotides	The genome is a variable-length string of characters; predefined signal sequences, analogous to promoters, terminators or start–stop codons, are used to detect genes; point mutations, indels and rearrangements can be simulated in a realistic manner	• The evolution of non-coding DNA and gene number[140] • The evolution of the size and topology of gene networks[126,127] • Gene network inference[157,158]

*Many formalisms have been proposed to represent the genome, each with strengths and weaknesses. The appropriate formalism strongly depends on the question of interest. Here, we focus on the approaches that are most directly comparable to *in vivo* microbial evolution experiments (that is, approaches for which the genome comprises several genes).

specific functions that were unnecessary in the selective environment[91,93]. However, further analyses also revealed mutations in genes that are not so directly related to the selective conditions, including those that are involved in regulatory pathways[5,47,48,92,94]. Evolution experiments in which bacteria are propagated in the presence of antibiotics represent one of the typical cases of selection that favours mutations in specific genes, including those encoding antibiotic-modifying enzymes, efflux pumps or cellular targets of the drugs[52,53,95]. However, even in these cases, it is difficult to consider these evolutionary pathways as simple local changes, for several reasons. First, changes that increase resistance often impose fitness costs[96] that in turn generate new selective pressures on global phenotypes such as growth and so can lead to diverse compensatory mutations[95]. Second, whole-genome sequencing of evolved resistant mutants revealed mutations in global regulators of gene expression[52]. Third, resistance mutations can have pleiotropic effects that lead to changes in other phenotypes[97], including virulence[95] and global gene expression[98–100]. Fourth, bacterial cell death, which is induced after antibiotic–target interactions, involves complex metabolic and regulatory networks[101], thereby imposing selective pressures on these networks in order for resistance to emerge.

Changes in expression profiles

The past decade has witnessed a major breakthrough in experimental evolution, with the application of high-throughput transcriptomics and proteomics. These techniques have revealed profound and parallel changes in the global gene expression profiles and metabolic pathways in evolved clones[17,31,33,35,45,63,72,102–104], highlighting the importance of modifications in regulatory and metabolic networks during evolution. Moreover, the use of new genome-sequencing technologies facilitates the identification of the underlying mutations[33,52,53,62,92,94,105]. Mutations have been identified in genes encoding global transcriptional regulators in almost every evolution experiment in which the mutations have been analysed, accounting for these global expression changes.

Owing to the availability of many tools, mutant strains and databases for *E. coli*, evolution experiments using this species as the ancestor were among the first targets of such analyses. Global transcription profiling and proteomics have been applied to the long-term evolution experiment (LTEE), which uses *E. coli* as the ancestor[12,46,93,102]. In this experiment, which is the longest-running evolution strategy, 12 populations have been propagated by daily serial transfer from a common ancestor in a defined minimal glucose medium for more than 50,000 generations[5,12]. Analysis of changes in the expression profiles of evolved clones compared with the profile of their common ancestor has enabled the identification of adaptive mutations in genes encoding global regulators that control two of the largest bacterial regulatory networks[5], the stringent response and DNA supercoiling, with these mutations occurring early in the experiment. Mutations in the same genes, including *spoT*, *topA* and *fis* (encoding bifunctional (p)ppGpp synthase–hydrolase, DNA topoisomerase 1 and DNA-binding protein Fis, respectively), were

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repeatedly discovered in most of the 12 independent populations of the LTEE[64,93,102]. Novel global regulators have also been discovered through such studies, including tRNA dihydrouridine synthase B (DusB), which appears to be involved in controlling DNA supercoiling[64]. Evolution of *E. coli* cells in chemostats leads to the spread of evolved clones with mutations in *spoT*, *rpoS* (encoding the stress-specific alternative RNA polymerase (RNAP) σ-factor, σ^S) and *hfq* (encoding an RNA chaperone that is essential for the regulatory effects of small RNAs)[106,107]. As a further mutational target, RNAP has been modified during adaptation of *E. coli* cells in minimal media[47,92]. Under these conditions, the mutations that confer most of the benefit to these adapted populations affect genes encoding subunits of RNAP[108] but, strikingly, not the subunits encoding the more specific functions. The evolved RNAP enzymes exhibit altered kinetic parameters, increased transcription elongation rates and an altered chromosomal distribution, causing a drastic reprogramming of gene expression that is probably specific to adaptation to minimal media.

Molecular analyses of the evolution of cooperation and social traits also highlighted the importance of regulatory networks for the adaptive abilities of microbial cells. A first set of studies investigated adaptive-radiation events during experiments in which *Pseudomonas fluorescens* ancestor cells were evolved in static structured broths. During these evolution experiments, cooperative cells (called WS cells, for wrinkly spreader) emerged with the new ability to colonize the air–liquid interface of the selective environment[23]. The radiation event leading to WS cells was based on two main cellular features. The first feature is the presence (in the ancestral genome) of an operon of previously unknown function, called *wss*, that is involved in the production of a cellulose polymer which is necessary to colonize the air–liquid interface[54]. The second feature is the emergence (in the ancestral cells) of mutations in three different regulatory modules, two of which were previously unknown and all of which contain a cyclic diguanylyl cyclase-encoding gene. These mutations result in the WS cell-specific constitutive expression of the *wss* operon[24]. A second set of evolution experiments investigated the evolutionary transitions from an ancestral cooperator (WT) of the differentiating bacterium *Myxococcus xanthus* to a developmentally defective cheater (OC) and back to a socially dominant cooperator (PX). Fourteen mutations occurred during the WT–OC transition[105], but only one additional mutation was fixed during the restoration of social behaviour in PX. This mutation is associated with a novel global transcription profile[104]. It affects a gene encoding a previously unidentified small RNA, which has since been shown to be a major regulatory checkpoint controlling the transition of *M. xanthus* from growth to development[109].

Although these observations are not so surprising, they bring to mind the tinkerer metaphor[110] by showing that evolutionary innovation relies more on the reorganization of molecular components than on the recruitment of new ones (note, however, that most evolution experiments are designed to avoid horizontal gene transfer, in contrast to natural environments). Indeed, organisms evolve complex adaptive traits by globally fine-tuning gene expression rather than by locally restructuring pathways that are involved in specific traits (FIG. 3). The identification of these adaptive mutations therefore enables the investigation of network architecture and of the rewiring that sustains fitness improvement. Along with the emergence of systems biology, these results emphasize the role of networks in organismal adaptation. However, whereas systems biology puts the stress on networks as a structurally static source of adaptation through the dynamics of their nodes, experimental evolution also emphasizes the role of networks as dynamic structures that can be targets of natural selection, thereby providing the organism with a high degree of evolvability.

Evolution of regulatory networks

In vivo and *in silico* evolution experiments have provided invaluable insights into the dynamics of regulatory networks, and four characteristics of the network architecture have been particularly investigated: pleiotropy, epistasis, plasticity and modularity.

Adaptive mutations affecting global regulators exhibit pleiotropic effects (FIG. 3). Even if these mutations are beneficial on average, they are likely to have maladaptive side effects. Further adaptation might therefore involve compensatory changes that contribute to the reduction of these side effects. During the adaptive radiation of *P. fluorescens*, a single mutation conferring the WS phenotype alters the expression of more than 50 proteins[103], none of which is required for the adaptive phenotype itself. Moreover, the emergence of WS cells is associated with a decrease in catabolism[111], but this may be partly recovered after prolonged propagation in the same environment owing to potential compensatory mutations. Similarly, evolution of *E. coli* in minimal lactate medium and in minimal glycerol medium revealed a two-step adaptive process, with large pleiotropic effects occurring first, including many transcriptional changes, followed by compensatory modifications that ensure most genes return to their baseline transcription level[45].

In the *E. coli* LTEE, adaptive changes in global regulators involve pervasive epistatic interactions. Deleting the *crp* gene, encoding the global regulator cyclic AMP receptor protein (Crp; which has so far been unchanged during the evolution experiment), results in much more drastic changes in both the growth rate and the global transcription profile in two independent evolved clones than in the ancestor[46]. This parallel expansion of the Crp regulon results from epistatic interactions between *crp* and mutations affecting other regulatory loci, thereby restructuring the connections between regulators. Moreover, negative epistatic interactions, resulting in declining rates of adaptation over time, have been detected between the first five beneficial mutations substituted in one evolving population, including some interactions affecting global regulator genes[112]. Such epistatic interactions between regulatory mutations also affect the evolutionary fate of a population, influencing the substitution of further beneficial mutations[113]. In an unrelated experiment in which *E. coli* evolved in a

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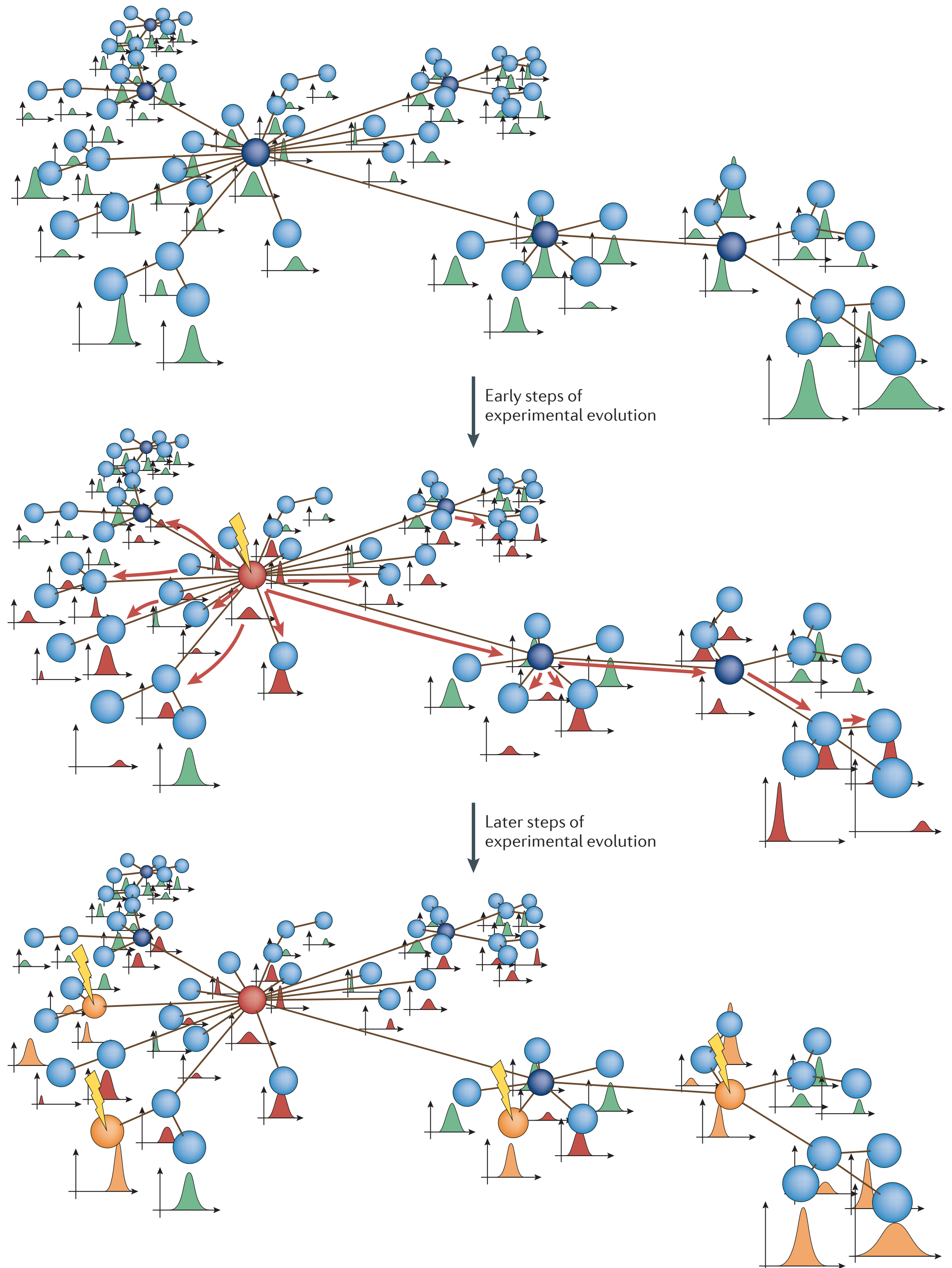

Figure 3 | ***In vivo* evolution of regulatory networks.** The structure of the network is hierarchical in the ancestral strain, with hub-encoding genes (dark blue circles) controlling many lower-level regulatory and target genes (light blue circles). The expression profiles of each gene as a function of growth conditions are shown as green curves, with the sum of all curves defining the phenotypic landscape of the individual. The early steps of experimental evolution are characterized by the occurrence of a mutation (yellow arrow) within a hub-encoding gene (red circle), which subsequently shows altered expression. These changes diffuse (red arrows) inside the entire connected network and affect the expression (red curves) of many genes. The overall effect is beneficial, but there are many pleiotropic effects. Later during evolution, compensatory mutations that correct for the deleterious effects of the early mutation affect lower-level genes (orange circles) in the regulatory network and almost restore their expression as well as the expression of some target genes (orange curves). This figure highlights the evolvable structure and organization of regulatory networks.

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medium containing glucose and acetate, both the fitness and phenotypic effects of an adaptive change in a global regulator were dependent on genomic background and population composition[114]. Moreover, adaptation of *E. coli* to high temperature demonstrates that there are at least two different evolutionary pathways that are shaped by epistatic interactions between beneficial mutations affecting global regulators[62].

Evolution experiments using mutants with artificially disturbed networks as ancestors further highlight the plasticity of cellular networks, as recovery often involves fast activation of unused pathways. This has been shown with *E. coli* mutants lacking genes encoding key enzymes[31,33,34] or global regulators[32], and with mutants of *P. fluorescens*[24], *M. xanthus*[22] and *Saccharomyces cerevisiae*[35].

Such variation and fine-tuning of regulatory networks have also been detected directly in nature, either by comparing natural isolates of a bacterial species or by investigating bacterial evolution inside eukaryotic host cells. Indeed, polymorphisms have been detected in the nucleotide sequences of *rpoS* and *spoT*, and variations in the amount and/or activity of their respective encoded proteins have also been found in natural isolates of *E. coli*[115,116]. Moreover, the levels of σ^S are highly variable in sampled within-patient *E. coli* isolates[117]. In two recent studies, bacterial evolutionary dynamics in the natural environment of the human host was investigated by characterizing the phenotypic and genetic changes of a collection of *Pseudomonas aeruginosa* and *Burkholderia dolosa* isolates that were sampled over a period of 35 years[118] and 16 years[119], respectively, from patients with cystic fibrosis. Genome sequencing revealed that mutations affecting three of the most global regulatory genes in *P. aeruginosa* tended to occur at early time points, and mutations were also found in genes encoding a two-component regulator and a σ-factor in *B. dolosa*, emphasizing the crucial involvement of global regulatory networks during bacterial adaptation. Moreover, parallel molecular adaptive evolution, as seen in laboratory experiments, was also detected directly in patients[119].

The recurrent observation that phenotypic innovation often occurs through global perturbation of cellular networks, possibly followed by local compensatory mutations, challenges our understanding of network evolution. Networks are highly evolvable structures, but this ability is strongly linked to their organization and connectivity, which result from their evolution. *In silico* evolution provides powerful tools to generalize results from *in vivo* experiments and can help researchers to understand the rules behind their observations (FIG. 4). Many models, mainly using the 'network' formalism (TABLE 2), have been designed to study the origin of specific wiring structures. In particular, network modularity has been extensively studied, as its evolutionary origins and consequences remain obscure[120,121]. *In silico* network evolution in different environments revealed that modularity can spontaneously arise when there are repeated switches between different environmental conditions[122]. Moreover, the modular structure probably speeds up evolution under these conditions because it increases evolvability. Modularity is also supposed to increase robustness by protecting organisms against pleiotropic effects[121]. It may therefore arise when pleiotropic effects are specifically deleterious, such as when an organism acquires new functions but still needs ancestral ones[123].

In silico network evolution has also been used to investigate the genetic structures that are most likely to appear during evolution when the organism has to fulfil specific functional characteristics. Bistability has been a particular focus[124], as it is common in bacteria[125]. However, the evolutionary fate of a given network structure depends not only on the environmental conditions but also on the mutational process and the genotype–phenotype map[121]. Thus, the conclusions derived from models using the 'network' formalism need to be generalized. The 'sequence-of-nucleotides' formalism (TABLE 2), although more complex, enables realistic mutational dynamics, including large chromosomal rearrangements. Extension of this formalism to network encoding has been proposed, and experiments have shown that the overall network structure strongly depends on mutation patterns[126] and mutation rates[127].

Evo–evo: variability, robustness and evolvability

The ultimate roots of the observed innovations are the mutational events, including point mutations and rearrangements. By enabling comparison of known 'fossil' sequences over evolutionary time points, experimental evolution provides a direct measurement of substitution rates and biases. The LTEE was recently used to provide the most accurate calculation to date of a bacterial mutation rate[128]. The identification of all the synonymous mutations in the genome sequences of 19 evolved *E. coli* clones, sampled from the 12 replicate populations and representing a total of 300,000 generations summed over all populations, gave an estimate of 8.9×10^{-11} point mutations per base pair per generation, with a substantial GC-to-AT bias. Moreover, the same fossil records can be used to study the evolution of mutation rates and, in turn, strains with mutations in the DNA repair systems can be used as ancestors for evolution experiments to provide a better understanding of the effect of higher mutation rates on evolutionary processes. Finally, evolution experiments represent empirical tools in the otherwise almost exclusively theoretical debate on the evolution of robustness[30] and evolvability[90] — that is, the evolution of evolution, or 'evo–evo', debate.

Increased mutation rates have often been shown to evolve during evolution experiments[2,4,6,94,129,130], reflecting observations in nature[131]. Moreover, different mutation rates have been shown to affect the dynamics of evolution rates[132,133], of phenotypic evolution[134] and of genomic evolution[28,29]. Such trends can be easily explored *in silico* using the 'allelic' formalism (TABLE 2). Models have shown that mutator alleles can promote accelerated adaptation even if they are not fixed[135] and that moderate (10–100-fold) mutators can reach fixation. This effect depends on both population size and selection strength[136]. The gradual evolution of mutation rates has also been studied with digital organisms, leading to a theoretically optimal mutation rate[137], although this value depends on

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the structure of the fitness landscape[138] and, thus, on the genotype–phenotype map.

These results, as well as the speed of evolution observed in most experiments, raise the question of the trade-off between the ability of an organism to innovate and to reduce the mutational burden that is imposed by increased mutation rates. Indeed, the evolution of mutators can be thought of as an example of the more general process of indirect selection of a specific level of variability[139]. Because the mutational variability of the phenotype is multifactorial, this indirect pressure can shape not only the mutation rate but also the global genetic architecture and the properties of the genotype–phenotype map[90]. This was demonstrated in *in silico* evolution experiments in which direct selective pressures were precisely controlled, thus making indirect pressures more visible. First, *in silico* studies showed that the indirect selection of a specific variability level can shape the genome structure at the levels of gene number, genome size and amount of non-coding DNA[140], and also at the level of gene order[141]. In both cases, chromosomal rearrangements play a crucial part in the observed effects. Second, several studies investigated the subtle relationships between direct selection for fitness and indirect selection for both evolvability and robustness. During competition experiments between a fit digital genotype and a less fit but more robust genotype, high mutation rates result in "selection of the flattest" — that is, the more robust genotype — instead of the usual selection of the fittest[142]. Thus, the key to evolutionary success is not the initial fitness but rather the average fitness of the descendants. Robustness in the genotype–phenotype map compensates for high mutation rates by re-establishing a tolerable level of variability. In these conditions, under which innovations are not rewarded, the driving evolutionary force is indirect selection for robustness rather than direct selection for fitness. However, when beneficial mutations are allowed, the driving force can also be indirect selection for evolvability. Evolution of a gene-regulatory network through the 'string-of-pearls' formalism (TABLE 2) showed that evolution progressively shapes a genotype–phenotype map that allows for ever faster adaptation to environmental changes[143]: beneficial mutations become increasingly likely (FIG. 4), but robustness to the majority of mutations is maintained. Indirect selection for evolvability was also shown with the *E. coli* LTEE: two clones that have beneficial mutations and are known to have taken over the population have significantly lower fitness than two contemporary clones that nevertheless later went extinct[113]. Evolution has been repeatedly replayed from these four clones, and the two with lower fitness are the eventual winners owing to the fact that their genetic background provides greater potential for further beneficial mutations, thus revealing higher evolvability. Whether evolvability is hampered or promoted by robustness is still an open question[144]. Robustness may promote evolvability, as neutral mutations can pave the way for later evolutionary adaptation[145], as observed experimentally for evolution of thermotolerance in an RNA virus[146]. However, *in silico* studies showed that the effect of robustness on evolvability is time dependent[147]: genetic robustness fosters long-term evolvability but can be a counterproductive trait in the short term.

Indirect selection
Selection acting on a property of the mutational processes, genetic architecture or developmental system that is not adaptive by itself but facilitates adaptive phenotypic evolution.

Synthesis, weaknesses and challenges

Experimental evolution has provided many insights into the dynamics of evolutionary processes at all organizational levels. However, most of these results could possibly have been discovered by combining other approaches such as classical genetics, ecology, physiology and biochemistry. Nevertheless, the unique power of experimental evolution resides in two original abilities that provide a global picture of microbial evolution in a given experimental setting: the ability to impose selective conditions, which allows innovative scanning of the phenotypic landscape, and the ability to draw links between events that occurred in an adaptive landscape during a defined experiment. The relative pace of evolution, including the phenotypic and genotypic trajectories, can be followed in almost all experiments. Fast and substantial phenotypic changes occur systematically in most experiments and are sustained by a few molecular events. Moreover, the high degree of replication that is possible in microbial evolution experiments reveals that similar phenotypic changes are associated with genotypic events that are often similar at the component level but different at the molecular level (genetic parallelism but allelic divergence). Genetic parallelism may allow identification of the function of the molecular element that has been targeted by selection, whereas allelic divergence may drive differences in the phenotypic outcomes of the function that has been improved at the macroscopic level. In addition to genetic parallelism, phenotypic innovation may emerge after more specific changes.

Without exception, the data gathered from *in vivo* evolution experiments highlight the often claimed but rarely quantified evolvability of bacteria. These organisms are highly evolvable biological systems with astonishingly fast adaptation abilities. Moreover, their evolvability is rooted in the structure and plasticity of their cellular networks. Indeed, adaptive changes systematically include alterations in global regulators, thereby affecting regulatory networks, an observation that emphasizes their integrated properties and organization. As such, these results challenge our empirical view of systems control, because we — as conceivers and users — are more confident in systems with limited and manageable perturbations. Therefore, the integrated view of evolutionary processes that emerges from experimental evolution strategies requires a new understanding of bacterial dynamics that is even more integrated than the classical view, which considers networks as a sum of different pathways. It also raises novel questions: why does this structure emerge rather than a more modular structure with different functions that are more specialized? Are there network structures that are the most likely to emerge in given evolutionary conditions and, if so, what are they? And finally, how do networks evolve? These questions, and many others, cannot be approached directly by

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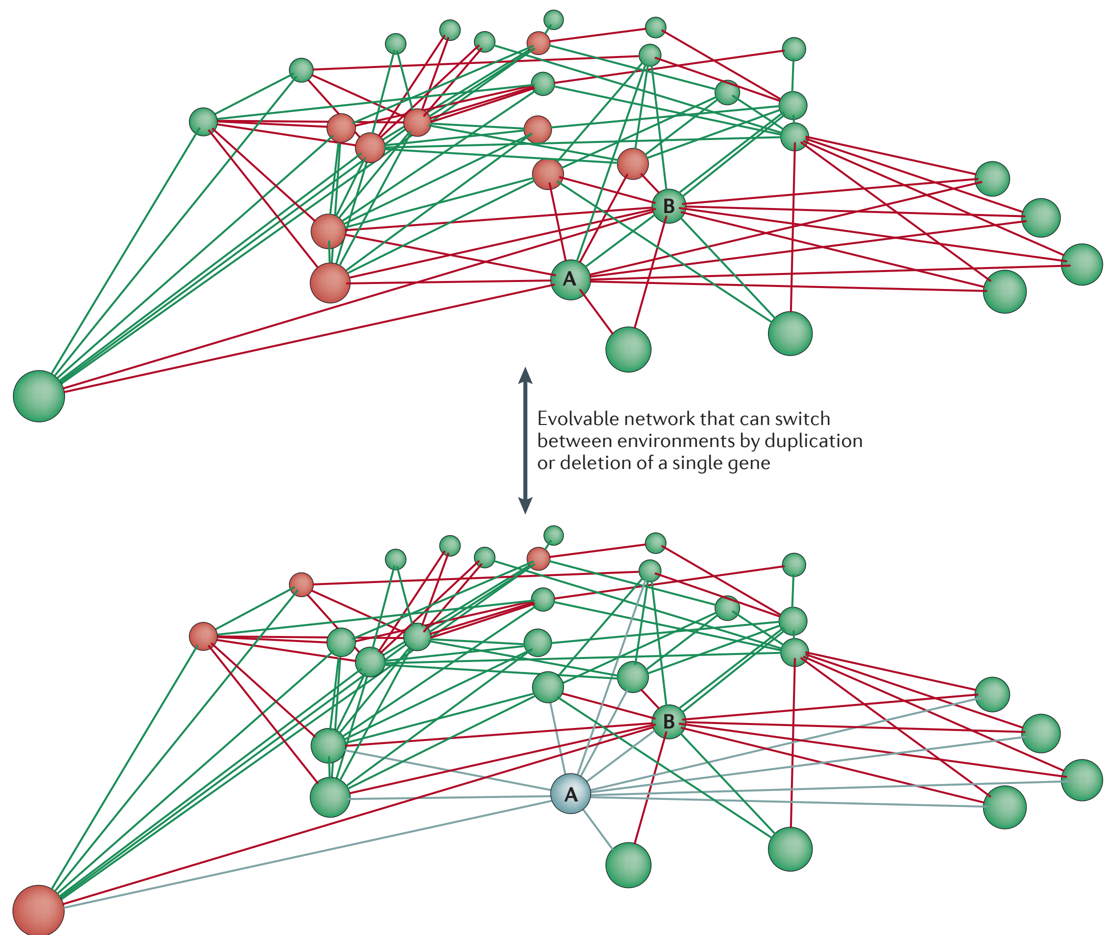

Figure 4 | ***In silico* evolution of regulatory networks.** Using the 'string-of-pearls' formalism, organisms were evolved in fluctuating environments consisting of a random switch between two different conditions. The evolved regulatory network (green and red lines representing activation and inhibition, respectively, and green and red nodes representing active and silent genes, respectively) shows a high degree of evolvability. Indeed, it can switch between the two situations by a single mutation: the deletion of gene A or its re-introduction to the genome by duplication of gene B (A being a copy of B). This single change modifies the equilibrium of the network, thereby switching many genes on or off. This experiment shows that, depending on the environmental conditions, regulatory networks can evolve toward highly evolvable structures. Figure is modified from REF. 143.

in vivo experimental evolution for three main reasons. First, the ancestor organisms have been in existence for, and have therefore been structured by, thousands of millions of years of macroevolution. Second, it would require evolution experiments in thousands of environments, with thousands of different ancestral strains and many replicates. Third, although bacteria divide very quickly, many years of laboratory work would be required to scan the entire regulatory landscape. However, these limits can be overcome by *in silico* evolution experiments. It is therefore now becoming urgent, after 20 years of independent development, for these approaches to converge to tackle the open questions of the structure, evolvability and robustness of microorganisms.

Increasing cooperation between *in vivo* and *in silico* experimental evolution is also likely to overcome the limits of both approaches. Without any contact with real life, digital genetics obviously risks losing its weak links with evolutionary biology and could therefore tackle more and more self-referential questions. This would severely limit its potential application in directing *in vivo* strategies to focus on the most helpful and relevant experiments, for example. Similarly, *in vivo* evolution experiments suffer from three major limitations, which are actually direct consequences of the main strengths of these experiments.

First, using precisely controlled laboratory conditions is undoubtedly a strength when it comes to addressing mechanistic issues. However, *in vivo* evolution experiments can therefore address only a small fraction of the entire complexity and variability of an ecosystem with its multiple interacting components, both at the biotic and abiotic levels. Although a minimal level of complexity has been shown to emerge in clonal populations even in the most simple and homogeneous environments, horizontal gene transfer and transitions between the multiple levels of organismal interactions (symbiosis, parasitism and commensalism) are examples of the many mechanisms

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that are known to be crucial in the dynamics of evolutionary processes but which are very difficult to study in test tubes or on plates. Moreover, a true reflection of the conditions that prevail in real ecosystems would require experiments involving many bacterial species, environments, replicates and time lengths. Second, using real and contemporary living organisms as ancestors for all intermediate and final evolved states is obviously a strength. However, it comes with important caveats, including the difficulties in exhaustively measuring and analysing the variations that occur during the experiment and the subsequent interpretation of these variations in terms of selective versus non-selective, direct versus indirect and strong versus weak evolutionary forces. Third, evolution experiments allow the direct observation of evolution in action. However, one can only climb on the bandwagon. Such experiments study the evolution of organisms with unknown past evolutionary histories and therefore can only speculate about the reasons why microorganisms are structured the way that they are and, thus, evolve the way that they do. Moreover, although it is relatively easy to analyse the winning individuals in an evolving population, it becomes a real challenge to identify and precisely characterize fit clones that eventually went to extinction, not to mention lineages that carried neutral or deleterious mutations.

Overcoming these limitations will be the greatest challenge for experimental evolution in the near future. As a specific example, introducing the ability to transfer genes horizontally would provide invaluable insights into the respective contributions of sex and mutation to evolutionary processes. In particular, it may then be possible to directly test whether adaptive changes would still target global regulatory genes. Increasing the complexity of evolutionary environments by reconstructing a complete ecosystem of interacting organisms will require new methodologies for growing multiple strains in multiple environments, or for evolving organisms in complex environments. Moreover, applications of omics techniques will also be needed to decipher the mechanisms that govern the entire phenotypic and genomic variation that practitioners observe in only a tiny fraction of evolving bacterial cells. Obviously, these challenges are related, and it will therefore also be necessary to better understand the interactions between them — that is, whether what we observe is only a consequence of the oversimplified environments in which the organisms evolve or whether it is the tip of an iceberg, the hidden part of which will allow us to discover the rules behind the evolutionary dynamics of regulatory networks.

After three decades, laboratory evolution is now at the end of its infancy. Similarly to other fields, evolution experiments have produced a huge amount of data for entire genomes, at the nucleotide, structure and expression levels. However, in contrast to other fields, these experiments produce data that can be both rooted to an available common ancestor and precisely followed through a line of descent for hundreds of generations. Despite these successes, *in vivo* experimental evolution has its own limitations, most of which are inherent to its methodology. Thus, as is the case for complementary fields such as systems biology, *in vivo* experimental evolution must combine with other areas, including biochemistry, ecology, microfluidics and high-throughput technologies. In the search for general evolutionary mechanisms, a much closer interaction between *in vivo* and *in silico* experimental evolution may create a virtuous cycle in which theories and experimental results progressively come closer and closer.

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Competing interests statement

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