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Experimental evolution as a tool to investigate natural processes and molecular functions

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Abstract

Experimental evolution of microbes has allowed evolutionary biologists to examine adaptive processes in real time, generating novel insights into fundamental laws of evolution. Much less appreciated is the potential of this approach to advance the understanding of microbial cells and molecular processes in complement of 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 experimental evolution can be employed to provide new light on microbial systems.

Highlights

- Experimental evolution (EE) can complement traditional molecular genetic studies on microbial systems.
- The diversity of EE approaches enabled progresses 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.

Glossary

Bow-tie network: architecture of a signalling network where one (or a few) core central regulator is controlled by multiple proteins (input signal) and controls multiple targets (output).

Collateral susceptibility: resistance to one antibiotic increases sensitivity to another.

Cross-resistance: resistance to one antibiotic increases resistance to another.

Experimental evolution: propagation of living organisms in a controlled environment – the laboratory or the field - for several generations (typically, from tens to thousands) allowing to witness the action of natural selection and to investigate evolutionary processes

Functional promiscuity: the ability of a protein to perform a secondary activity, often mediated by the inability to distinguish between target molecular substrates (metabolite, DNA, RNA, protein) that have a similar structure.

Fruiting body: multicellular, aggregative structures formed by myxobacteria during nutrient starvation and that contain spores resistant to heat, desiccation or freezing.

Mutators: bacteria with unusually high mutation rates, often as a result of loss of DNA repair genes or expression of error-prone DNA polymerases.

Noise in gene expression: stochastic variations in gene expression level over time and between isogenic cells growing in a homogeneous environment.

Pervasive transcription: describes the fact that large portions of genomes are transcribed, including intergenic regions.

58 **sRNA**: small non-coding RNA with regulatory function
59 **Sub-MIC concentrations**: conditions where bacterial growth is not completely stopped by
60 antibiotics.
61
62 **Outstanding questions**:
63 - Can we exploit mutations found in EE to characterise genes of unknown function?
64 - Can EE enlighten mechanisms slowing down the evolution of resistances?
65 - Can the EE of microbial communities bring new insights into their functional
66 characteristics?
67 - Can we use within-host EE to identify host factors (and other environmental factors)
68 shaping bacterial evolutionary trajectories during infection?
69 - Can EE reproduce the emergence of major intracellular symbiotic associations (such
70 as mitochondria in eukaryotic cells, plastids in the green lineage, association between
71 fungi and land plants, obligate endosymbionts of insects)?
72

73 **Experimental evolution: beyond evolutionary biology**

74 Artificial breeding of plants and animals played a key role in the maturation of Darwin's theory
75 of evolution by natural selection, but also in the rise of modern human societies (from
76 agriculture to recreational breeding of plants and pets). Non-professional evolutionary
77 biologists performing (sometimes unwittingly) evolution experiments have successfully
78 improved the yield of many biological processes. In contrast, although some evolutionary
79 biologists contemporary of Darwin turned to **experimental evolution** (EE; see **Glossary**), the
80 influence of this approach in the development of evolutionary biology only came to
81 prominence in the last few decades [1]. Applied to microbes with short generation times in
82 various conditions (Box 1), EE generated unique biological material (Fig. 1) that was used to
83 probe the evolutionary dynamics of microbial phenotypes [2, 3], to decipher the genetic bases
84 of adaptation [4] and to optimize microbial traits for industrial use [5].
85
86 In spite of these successes, many biologists remain unaware or unconvinced of the relevance
87 of EE approaches to their research programs. Typical criticisms of EE include the artificial
88 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. In this article, we argue that experimental evolution 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 experimental evolution 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 aiming at 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 on gene regulation, antibiotic resistance and host-microbiome interactions to illustrate how EE contributes to understand fundamental aspects of molecular biology and helps manipulating natural ecosystems. As 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 examples of discrepancies (Box 2), but also increasing evidence 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 interactions of transcription factors (TF) with DNA arise and evolve, why they are so hard to identify correctly, and how they form complex networks [13].

The co-option or modulation of bacterial regulatory responses provide a multitude of opportunities for adaptation to new environmental challenges. Indeed, most adaptive mutations in EE are found in regulatory regions or in regulatory genes [4]. Detailed molecular analyses of the associated transcriptional rewiring can reveal new components from signalling

121 networks, new connections between known regulatory components and new cross-talk
122 between signalling and metabolic pathways. These trends emerged from some of the very
123 early studies in experimental evolution [14, 15]. A prominent example includes the evolution
124 of a new lactose fermentation system in *Escherichia coli*, via mutations in the *egbA* β-
125 galactosidase and its regulator [16]. More recently, EE has uncovered a novel **sRNA** regulating
126 *Myxococcus xanthus* **fruiting body** development. *M. xanthus* responds to nutrient starvation
127 by generating fruiting bodies containing stress-resistant spores. This behaviour was lost during
128 EE in nutrient-rich liquid medium, but was subsequently re-evolved in alternating cycles of
129 starvation and non-starvation [17]. The point mutation responsible for the re-evolution of
130 fruiting-body formation was found in a previously un-annotated intergenic region, leading to
131 the discovery of the novel sRNA controlling this developmental pathway [18]. Another study
132 established a link between pyrimidine metabolism and the Gac/Rsm signalling pathway, a
133 major determinant of lifestyle switch in *Pseudomonas* spp. [19] that controls the production
134 of extracellular capsules in *Pseudomonas fluorescens*. Evolution of *P. fluorescens* under
135 fluctuating environments gave rise to a strain that produces a sub-population of capsulated
136 cells [20]. This phenotype is underpinned by a mutation that decreases pyrimidine
137 biosynthesis [21] and increases ribosome production [22]. That both increased ribosome
138 levels and functional Gac/Rsm signalling are required for heterogeneous capsule production
139 shows that central metabolism (pyrimidine biosynthesis) can alter the output of a two-
140 component signalling system [22].
141
142 The plasticity of regulatory regions and transcription factors observed in EE can be recruited
143 to understand fundamental properties of transcriptional control. Low affinity TF-DNA
144 interactions appear to play a critical role in the evolution of GRNs. Yona *et al*. [23] replaced
145 the *lac* promoter with random DNA sequences of the same size in *E. coli* and observed that
146 bacterial growth in the presence of lactose rapidly led to the evolution of functional
147 promoters. A single mutation in synthetic promoters was sufficient to induce substantial
148 expression in most cases (Fig. 2A). The rapid *de novo* evolution of promoters and the
149 **functional promiscuity** of transcription factors may explain the intriguing **pervasive**
150 **transcription** observed in bacterial genomes [24]. It may also explain the evolutionary
151 plasticity of gene expression in bacteria, since an initially weak binding of a TF on a regulatory
152 region can be reinforced by mutations in the DNA-binding domain. This was observed during

153 the re-evolution of motility in *P. fluorescens* following the deletion of the master regulator of
154 flagellar synthesis *fleQ* [25]. Under selection for motility, flagellin expression was restored
155 within 96h in a two-step process involving (i) the increased phosphorylation (and thus,
156 activation level) of a nitrogen-related transcriptional regulator with weak (promiscuous)
157 activity on flagellar genes and (ii) a switch-of-function mutation that re-directed
158 transcriptional activity of this regulator from nitrogen uptake to flagellar genes. The plasticity
159 of regulatory interactions can also be associated with **noise in gene expression**, which has
160 attracted interest in the recent years because it can contribute to phenotypic variability in
161 clonal populations [26]. Wolf *et al.* [27] showed that libraries of random promoters evolved to
162 produce GFP at high or intermediate expression levels had lower average noise than natural
163 *E. coli* promoters. This suggests that noise is a selected trait in nature. When the average
164 production of a protein is not optimal in a given environment, a broad (i.e. noisy) distribution
165 of gene expression levels increases the likelihood that some cells of the population express an
166 appropriate amount of this protein. Mathematical modelling suggests that noise can act as a
167 primitive form of gene regulation and pave the way towards more precise regulation [27].
168 Altogether, these studies illustrate that what may appear to be 'non-optimality' in
169 transcriptional regulation – functional promiscuity and noise – actually drives the evolution of
170 GRNs.
171
172 EE enables to directly probe systems-level properties of GRNs in an evolutionary context.
173 Witnessing how GRNs are gradually built by evolutionary tinkering (rather than by optimized
174 design) is key to understand their complex architecture and emergent properties, such as
175 robustness, resilience or evolvability [28]. In many cases, transcriptional changes induced by
176 genetic or environmental perturbations are reverted during the early steps of adaptation by
177 adaptive mutations in global regulators that restore cellular homeostasis [29-32]. These
178 results support the idea that transcriptional stability is essential for optimal fitness and can be
179 restored by minimal genetic modifications in global regulators, thus highlighting the resilience
180 of GRNs. However, the topology of GRNs architecture can also benefit bacterial evolvability.
181 Studying the evolution of the **bow-tie network** controlling flagellar production in *E. coli*, Ni *et*
182 *al.* [33] found that motility in a porous environment (the trait under selection in their
183 experiment) can increase via mutations in multiple target genes. Yet, all adaptive mutations
184 commonly modified the activity of a core sigma-factor checkpoint controlling flagellar gene

185 expression. By allowing multiple mutations in independent genes to target the same central
186 regulator of motility, the bow-tie architecture provides evolutionary flexibility for the fine-
187 tuning of bacterial behaviours. These examples show that EE is a powerful tool to analyse
188 evolutionary properties of GRNs.
189
190
191 **Antibiotic resistance**
192 The emergence of antibiotic resistant bacteria is a remarkable example of human-induced
193 evolutionary process. Resistant bacteria emerge systematically within a few years of the
194 introduction of every novel antibiotic. The consequences are dramatic: 700,000 deaths per
195 year, estimated to increase to over 10 million in the incoming decades (https://amr-
196 review.org/Publications). EE is particularly well-suited to decipher the mechanisms of
197 acquisition of antibiotic resistance given the timescale and intensity of selection in this
198 process. This has produced unexpected novel lines of research. For example, very early studies
199 on the EE of antibiotic resistance kick-started the study of microbial mutagenesis by
200 identifying the first *E. coli* **mutators** [34]. The simplicity of tracking resistant bacteria, the
201 societal relevance of the topic, and its implications to molecular genetics and physiology have
202 stimulated research on the mechanisms of acquisition of resistance, on multiple resistance,
203 and on the compensation of the fitness costs of resistance.
204
205 Most resistances studied from clinical isolates are strong, because of the obvious medical
206 interest of such cases. EE provides complementary information about resistance in controlled
207 setups where pathways to resistance can be tracked, selection forces tuned, and physiological
208 states controlled for. Notably, EE revealed the important role of low (below the minimum
209 inhibitory concentration; MIC) antibiotic concentrations on the evolution of resistance. These
210 concentrations can be encountered in many types of environments and do not result in cell
211 death, but provide sufficient pressure for the selection of resistant variants and even the
212 evolution of novel mechanisms of resistance [35]. They also accelerate the acquisition of
213 resistance by selecting for mutators [36], and increasing the rates of horizontal gene transfer
214 [37]. Lindsey *et al.* [38] evolved hundreds of populations of *E. coli* under variable rates of
215 increase in concentration of rifampicin. By assessing fitness of genetically engineered
216 combinations of mutations from isolates evolved under low rates of environmental change,

217 they could show that certain genotypes were evolutionarily inaccessible to evolution under
218 rapid environmental changes. Wistrand-Yuen *et al.* [39] then showed that *Salmonella enterica*
219 exposed to **sub-MIC levels** of streptomycin evolved high-level resistance via mechanisms
220 different from those observed under above-MIC conditions. Sub-MIC resistance evolved
221 through small-effect mutations that combined to confer high-level resistance.
222
223 Antibiotics stimulate a set of core responses in bacterial physiology [40], which contributes to
224 explain why resistance to one antibiotic can change the cell's susceptibility to others [41].
225 Several recent studies detailed the network of **cross-resistance** and **collateral susceptibility**
226 resulting from EE of resistance to each of a large range of antibiotics [42-44]. Evolution
227 involving cross-resistance and collateral sensitivity is frequently convergent in *E. coli*, meaning
228 that one can predict to a reasonable extent the antibiotic resistance phenotypes from the
229 genome sequences of the laboratory-evolved lines [45]. Treatments based on alternating
230 drugs with compatible collateral sensitivity profiles could thus be more efficient and lead to
231 slower development of resistances (Fig. 2B; [44]). Several of these studies observed that
232 populations adapted to resist to aminoglycosides show systematically lower fitness in the
233 presence of other types of antibiotics. In molecular terms, this could be the consequence of
234 selection for alterations in the inner membrane potential reducing the uptake of
235 aminoglycoside-related antibiotics, which would simultaneously lower the activity of efflux
236 pumps using proton-motive force [42]. Interestingly, the comparison of interaction networks
237 evolving in conditions of weak and high antibiotic concentration revealed that the strength of
238 selection shapes the acquisition of resistance: cross-resistance tends to be stronger under
239 higher antibiotic concentrations [46]. The stochasticity of evolutionary trajectories leading to
240 resistance to one antibiotic can influence the occurrence of collateral sensitivity [47], calling
241 for a careful assessment of the robustness of this phenomenon when wanting to exploit it in
242 a clinical setting. Together with the studies on the effect of sub-MIC conditions, this suggests
243 that the path towards resistance, involving exposure to high or low antibiotic concentrations,
244 will shape the network of collateral effects of antibiotics.
245
246 The many evolution experiments detailing the mutational landscape of antibiotic resistance
247 have systematically revealed rapid partial compensatory evolution of the initial fitness costs
248 of resistance [48-50]. Compensation facilitates the spread and fixation of antibiotic resistant

249 lineages. For example, rifampicin resistance in *Mycobacterium tuberculosis* is costly in
250 laboratory-derived mutants, but multidrug-resistant clinical strains often show no fitness
251 defects [51], because of compensatory mutations [52]. The study of mutants arising in *E. coli*
252 in the presence of fluoroquinolones showed that compensation of fitness cost associated to
253 antibiotic resistance can lead to bacteria that are as fit as the wild-type susceptible bacteria in
254 the absence of antibiotics [53]. Prolonged colonization of chickens with fluoroquinolone-
255 resistant *Campylobacter jejuni*, a somewhat less controlled evolution experiment, revealed
256 that resistant bacteria were also fitter pathogens [54]. Genes that simultaneously increase
257 virulence and resistance may actually be quite common. They were observed in several other
258 pathogens, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Vibrio cholerae*
259 [55]. Even costly resistance mutations can coexist with other genotypes for hundreds of
260 generations when their high adaptive potential counteracts their initial cost [56]. This means
261 that a multi-step process of slow accumulation of mutations conferring resistance and cost
262 compensation, as expected under sub-MIC conditions, can result in bacteria that are both
263 resistant and fit. This process is strongly dependent on the genetic context of the mutations
264 (epistasis) [57-60]. For example, a comparison of streptomycin and rifampicin double-resistant
265 *E. coli* with single-resistant clones obtained though EE showed that low-fitness double-
266 resistant bacteria compensate their cost faster than single-resistant strains thanks to the
267 acquisition of compensatory mutations with larger effects [61]. Surprisingly, some mutations
268 only compensate for double resistance, being neutral or deleterious in single-resistant
269 backgrounds. This means that multiple resistances may not rapidly go away with pauses in the
270 use of the corresponding antibiotics.
271
272 **Host-microbe interactions**
273 Plants and animals are persistently inhabited by microbes, whose contribution in host health,
274 nutrition and development is increasingly recognized [62, 63]. Elucidating the functional
275 mechanisms and evolutionary potential of host-microbiome interactions is crucial to
276 manipulate this ecosystem and improve host health.
277
278 Most bacterial pathogens have the remarkable ability to alternate between external
279 environments and specialized host niches. Rapid and coordinated shifts in metabolism,
280 physiology, and virulence factor production in response to environmental changes are

281 orchestrated by multilayered and highly complex circuitries that are very difficult to decipher
282 [64]. Adaptation to new hosts, which is generally very rapid in laboratory conditions [65], has
283 the potential to reveal new components of virulence pathways. This was recently highlighted
284 in an evolution experiment aiming at adapting the plant pathogen *Ralstonia solanacearum* to
285 different host plants [66]. Beneficial mutations improving *in planta* colonization mainly
286 occurred in a gene, which was named *efpR* (for enhanced fitness in plants). The *efpR* gene was
287 shown to encode a transcriptional regulator acting as both a central player of the
288 *R. solanacearum* virulence network and a global catabolic repressor down-regulating the
289 expression of multiple metabolic pathways [67]. Experimental adaptation of *R. solanacearum*
290 to a non-host legume further identified other components of the *efpR* pathway [68]. Although
291 the genetic bases of virulence in *R. solanacearum* had been amply dissected [69], this pathway
292 had not been previously identified, illustrating how EE coupled with genome resequencing
293 allows identifying novel molecular players of biological functions.
294
295 Many ecological transitions towards pathogenic or mutualistic symbiosis include an initial
296 acquisition by horizontal gene transfer (HGT) of mobile genetic elements (MGE) that can
297 provide complex novel traits in a single event of transfer [70, 71]. Profound changes in lifestyle
298 may require the remodeling of the metabolic and signaling networks in the recipient genome,
299 a process that may take hundreds to millions of years *in natura* [72]. The study of this process
300 by the analysis of extant microorganisms can be complemented by EE to pinpoint specific
301 molecular mechanisms that ensure the full expression of acquired traits. This is illustrated by
302 the study of nitrogen-fixing legume symbionts (rhizobia), which evolved through the
303 acquisition of a set of essential symbiotic genes [73]. An experiment was designed to evolve a
304 plant pathogenic bacterium (*R. solanacearum*) into a legume symbiont under plant (*Mimosa*)
305 selection pressure. (Fig. 2C). This experiment showed that control mechanisms developed by
306 the host progressively shape bacteria via the multistep selection of compatible bacterial traits
307 [74]. The gradual activation and improvement of the first symbiotic properties, *i.e.* the
308 induction of root nodules where bacteria fix nitrogen and the infection of these nodules,
309 occurred via the inactivation of the *R. solanacearum* pathogenic type III secretion system and
310 via regulatory rewiring [75-77], demonstrating the requirement of post-HGT modifications to
311 achieve symbiosis in the case of horizontal transfer between distantly related bacteria. Upon
312 experimentally replaying rhizobia evolution, a genetic mechanism was discovered to

313 accelerate symbiotic evolution [78]. Evolved *R. solanacearum* underwent a transient
314 hypermutagenesis stage that occurred at every inoculation cycle before the cells entered the
315 plant. Investigating the role of *imuABC* error-prone DNA polymerases present on the
316 transferred symbiotic plasmid provided evidence that this mutagenesis cassette is expressed
317 in stress conditions (outside the host plant), thus increasing genetic diversity and offering
318 more phenotypic diversity to plant selection. *imuABC* cassettes were found on *c.a.* 50% of the
319 symbiotic plasmids supporting the hypothesis that this hypermutagenesis mechanism has
320 facilitated the evolution of new rhizobia *in natura*. After 400 generations, mutualistic nitrogen
321 fixation was not achieved, possibly because time was too short. Yet another evolution
322 experiment performed with the natural symbiont of *Mimosa* (*Cupriavidus taiwanensis*)
323 showed that, thanks to host sanctions occurring at the post-infection level, rare nitrogen-fixing
324 symbionts (that may arise via mutation during evolution) progressively invade a population
325 dominated by non-fixing bacteria, with a probability that depends on ecological factors [79].
326 This provided a better understanding of the spread of the mutualistic trait during natural
327 evolution [73].
328
329 Plant and animal microbiomes are composed of complex and dynamic bacterial consortia
330 whose interspecific interactions have implications for the host [80]. The importance of
331 bacterial antagonism for the evolution of infection was demonstrated in a tri-partite
332 interaction between *Caenorhabditis elegans* and two bacterial pathogens. In a first EE, King *et*
333 *al.* [81] showed that mildly pathogenic bacteria (*Enterococcus faecalis*) living in worms rapidly
334 evolved increased competitiveness against a more virulent pathogen (*Staphylococcus aureus*).
335 This reduced the mortality caused by *S. aureus* infections. The mechanistic basis for protection
336 was an increased production by *E. faecalis* of antimicrobial reactive oxygen species directly
337 affecting pathogen growth. Although this broad-spectrum defense mechanism was not novel,
338 it showed that microbes living within a host can become mutualists in response to infection
339 by other pathogens. A subsequent EE showed that microbe-microbe interactions within hosts
340 can drive the evolution of pathogens. To limit *E. faecalis* colonization, which exploits the costly
341 siderophores of *S. aureus*, the latter evolved to produce less siderophores [82]. Since
342 siderophore production contributes to virulence by improving pathogen growth in iron-
343 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 the conditions driving phenotypic adaptation in a number of model biological systems. We expect that many more discoveries will follow. For practical purposes, characterisation of adaptive mutations has usually focused on known genes and pathways. A large number of adaptive mutations found in genes of unknown function remains to be analysed, representing a challenging untapped reservoir of new discoveries. The development of cross-experiments databases (such as the recent ALEdb [84]) may help identifying promising candidate genes to initiate such studies. Moreover, the introduction of novel non-model bacteria allows EE to tackle different biological questions, *e.g.* studying the evolution and functioning of microbial communities (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 remarkable interest to use EE as a tool to test hypothesis 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 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’, tremendously 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]. With continuously decreasing sequencing cost, the EE field is therefore ripe for appropriation by molecular microbiologists coming with imaginative selective regimes, original microbial strains or communities, and

376 novel biological questions. More generally, the adoption of EE by a wider community of
377 microbiologists could accelerate the march towards the much-needed synthesis of molecular
378 and evolutionary approaches [87-89].
379
380
381 **Box 1: The many faces of experimental evolution**
382 Experimental evolution can be performed under a wide range of experimental settings
383 adapted to the biological question of interest. Details on the design of these studies were
384 reviewed elsewhere [3, 90]. As an attempt to classify the most common EE practices, one can
385 distinguish levels of complexity along two criteria: environmental conditions and starting
386 biological material (Fig. 1).
387 Starting biological material: Wild-type bacterial strains are commonly used to start evolution
388 experiments. Although in theory any cultivable strain can be used, most works focus on fast-
389 growing genetically tractable model bacteria. Other studies employ modify-and-evolve
390 approaches where genetic engineering is used to delete [32], introduce a trait [23, 27, 75] or
391 modify a gene or the whole genome [91, 92], and is followed by EE to understand how the
392 system evolves. These approaches profit enormously from the recent developments in
393 synthetic biology and CRISPR-based technologies. Comparative evolution studies are typically
394 performed by evolving several independent lines from the same ancestor in parallel, but can
395 also involve different strains/species exposed to the same conditions [93]. Complexity in
396 starting material can be increased by putting together different bacterial species [94, 95], or
397 complex communities [96]. In these cases, experimentalists can follow either the evolution of
398 one of the organisms or the co-evolution of multiple organisms.
399 Environmental complexity: Following the trends set by the long-term evolution experiment
400 (LTEE), most microbial evolution experiments are performed in very simple growth conditions,
401 e.g. in shaken Erlenmeyer flasks [97] or in chemostats [98], with a variety of volumes, time
402 delay and volume of transfer between subsequent cycles (flasks) or dilution rates
403 (chemostats). A number of studies used more complex environments. Spatially structured
404 environments are generated in static liquid cultures (creating an oxygen gradient [99]), on
405 solid supports within liquid medium [100] or on solid surfaces (agar plates [25]). In this case,
406 micro-organisms deplete resources locally, leading to differences between patches. Growth
407 on solid media or in liquid meta-populations also helps manipulating genetic assortment

408 between neighbouring cells, a condition often used to explore the evolution of social
409 behaviours [101-103]. The strength of selective pressures can be adjusted during the
410 experiment by varying antibiotic concentrations in a continuous culture [58], creating
411 chemical gradients in agar plates [104], or by varying conditions between successive growth
412 cycles [20, 43]. Finally, some studies mimic natural conditions more closely by employing more
413 complex settings, such as eukaryotic hosts, to study the evolution of mutualists or pathogens
414 [66, 77, 81, 105-107].
415
416 **Box 2: Divergences between EE and natural evolution**.

417 Many EE studies identified patterns of molecular evolution with large excesses of non-
418 synonymous adaptive mutations, whereas natural populations systematically show a
419 predominance of synonymous substitutions caused by purifying selection on protein
420 sequences [108]. This may result from the joint effects of the simple continuous unidirectional
421 selective pressures applied in many EE together with the use of conditions free from most
422 other constraints that bacteria endure in natural environments. The same reasons may explain
423 why core genes tend to accumulate few substitutions in natural populations, but evolve faster
424 in the LTEE [109]. The contrast between evolutionary patterns is particularly striking for the
425 RNA polymerase gene *rpoB* that often accumulates adaptive mutations in *in vitro* EE
426 experiments but is extremely conserved in natural evolution [4]. Interestingly, a recent *E. coli*
427 EE study in the mouse gut, a more natural environment, showed lower rates of evolution and
428 no mutations in *rpoB* [110]. Similar discrepancies were found by a study where *P. aeruginosa*
429 adaptation to the airways of cystic fibrosis patients during over 200,000 generations resulted
430 in limited genetic diversification. In contrast with *in vitro* EE, the *in vivo* process revealed an
431 initial period of adaptive mutations followed by a period with the more usual pattern of
432 dominance of purifying selection [111]. Another reason for the excess of non-synonymous
433 adaptive mutations in EE is the lack of sexual exchanges with distant strains or species in most
434 EE setups. This prevents the income of adaptive changes by horizontal gene transfer and
435 results in adaptive mutations touching key processes that are highly conserved in nature. For
436 example, many traits are lost in EE because they are costly in simplified setups, but they are
437 under selection – and thus conserved – in nature [112]. A striking example of this contrast is
438 given by the frequent evolution of mutators during phage-bacteria co-evolution in simple

439 experiments, which was not observed in complex environments closer to natural conditions
440 [113, 114].

441

442 **Box 3: Parallels between EE and natural evolution**.

443 The use of more complex setups in EE has found interesting parallels with analogous processes
444 in natural history. For example, diversification of *M. xanthus* during EE led to genetic diversity
445 close to those identified in natural populations sampled from small parcels in the soil [115].
446 EE of *Burkholderia cenocepacia* in biofilms revealed a wealth of mutations associated with its
447 adaptation and diversification of which four broad classes were also found in clinical isolates
448 of *Burkholderia dolosa* and *P. aeruginosa* cystic fibrosis patients, suggesting a parallelism
449 between adaptation to the biofilm lifestyle and lung colonization [100]. A recent study aimed
450 at comparing directly the patterns of evolution of resistance to colistin in *P. aeruginosa* using
451 both laboratory EE and the analysis of four clinical isolates from a single cystic fibrosis patient
452 (sampled within a period of three months) [60]. This revealed a complex, multistep adaptation
453 process requiring epistatic mutations in several loci where parallels between the natural and
454 experimental processes could be identified: all resistant mutants were mutators and the
455 evolution of resistance occurred through mutations in *prmB*, part of the PrmAB two-
456 component system. This shows that processes at comparable time scales requiring a relatively
457 straightforward adaptation process can reveal significant parallels. Another study combining
458 detailed phenotypic characterization and mathematical modelling showed that high
459 mutational supply, influenced by population and bottleneck sizes, was a key parameter
460 favoring parallelism between laboratory and natural evolution of ciprofloxacin resistance in *E.*
461 *coli* [116].

462 More complex adaptation processes were investigated by evolving three clones of
463 *Lactococcus lactis* from a plant isolate to the dairy niche [117]. Gene expression differences
464 between the parental and the dairy strain were maximal at an operon encoding an ABC
465 transporter that was 350 times more expressed in the dairy strain. Interestingly this operon
466 was also expressed at higher level in two of the three adapted strains. An even more radical
467 EE, leading to a change in lifestyle from a plant pathogen to a rhizobial mutualist upon
468 acquisition of a large plasmid carrying the symbiosis genes and after a few hundreds of
469 generations (see Main Text), also showed striking parallels to the natural process that took

place over more than 10 million years [118]. In spite of 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 very little signal 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 works show that EE reveals significant parallels to natural history when it mimics key conditions of the natural processes.

Figure legends:

Fig. 1: Experimental evolution: a source of biological material available for phenotypic and genotypic analysis.

Traditionally, only extant or very 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 provides data that can be compared with that of EE. Points represent available naturally- or experimentally-evolved bacterial clones/populations.

Fig. 2. Selected evolution experiments having contributed to advances in gene regulation (A), antibiotic resistance (B) and host-microbe interactions (C).

A. Evolution of promoters [23]. Left: The *lac* promoter was replaced by random sequences in *E. coli*. Bacteria were evolved by serial dilutions in 0.05% glycerol (utilized by the strain) and 0.2% lactose (originally not utilized). Right: Following laboratory selection, ~60% of promoters having acquired a single mutation (black star) exhibit on average 50% of the wild-type (WT) activity.

498 B. Evolution of collateral sensitivity and its use to design new therapeutic strategies [44]. Left:
499 Bacteria selected to resist a given antibiotic (X) reproducibly display an increased (cross-
500 resistance), unchanged or decreased (collateral sensitivity) resistance to other antibiotics
501 (*e.g.*, A) compared to their WT ancestor. Right: Patterns of collateral sensitivity can be
502 exploited by cycling antibiotic treatments that accelerate eradication of bacterial pathogens.
503 CFU: colony forming units.

504 C. Evolution of new legume symbionts [77, 78]. The symbiosis plasmid of *Cupriavidus*
505 *taiwanensis* was introduced into *Ralstonia solanacearum*, generating a non-nodulating proto-
506 rhizobium (Nod^-) that was further evolved using serial cycles of co-culture with *Mimosa*
507 *pudica*, the natural host of *C. taiwanensis*. The symbiosis plasmid possesses the essential *nod*
508 and *nif/fix* genes required for nodulation and nitrogen fixation. In addition, it contains *imuABC*
509 genes encoding stress-responsive error-prone DNA polymerases that transiently elevated the
510 mutation rate of bacteria growing in the rhizosphere. In only 16 cycles (*c.a.* 400 generations)
511 the ancestral proto-rhizobium, which was only able to induce root hair curling (Hac^+) allowing
512 the formation of infection sites, successively acquired the capacity to enter the root and form
513 nodules (Nod^+), extracellularly infect nodules (E-Inf^+), intracellularly invade nodules (I-Inf^+) and
514 massively invade nodule cells (I-Inf^{++}), via genome remodeling. Stars symbolize mutations.
515 Bacteria are represented in blue in nodules. Adapted from references [74, 78].

516

517 **Fig. 1 for Box 1: Experimental settings in EE**

518 Examples of experiments using biological material or environments exhibiting increasing
519 levels of complexity.

520

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529
530
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Figure 1 (revised)

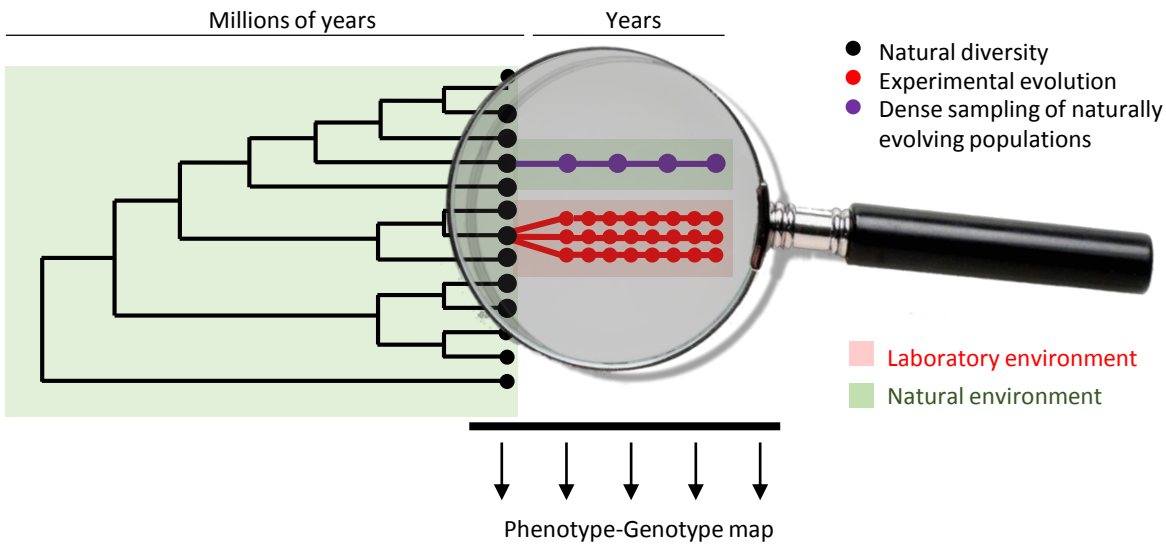


Figure 2 (revised)

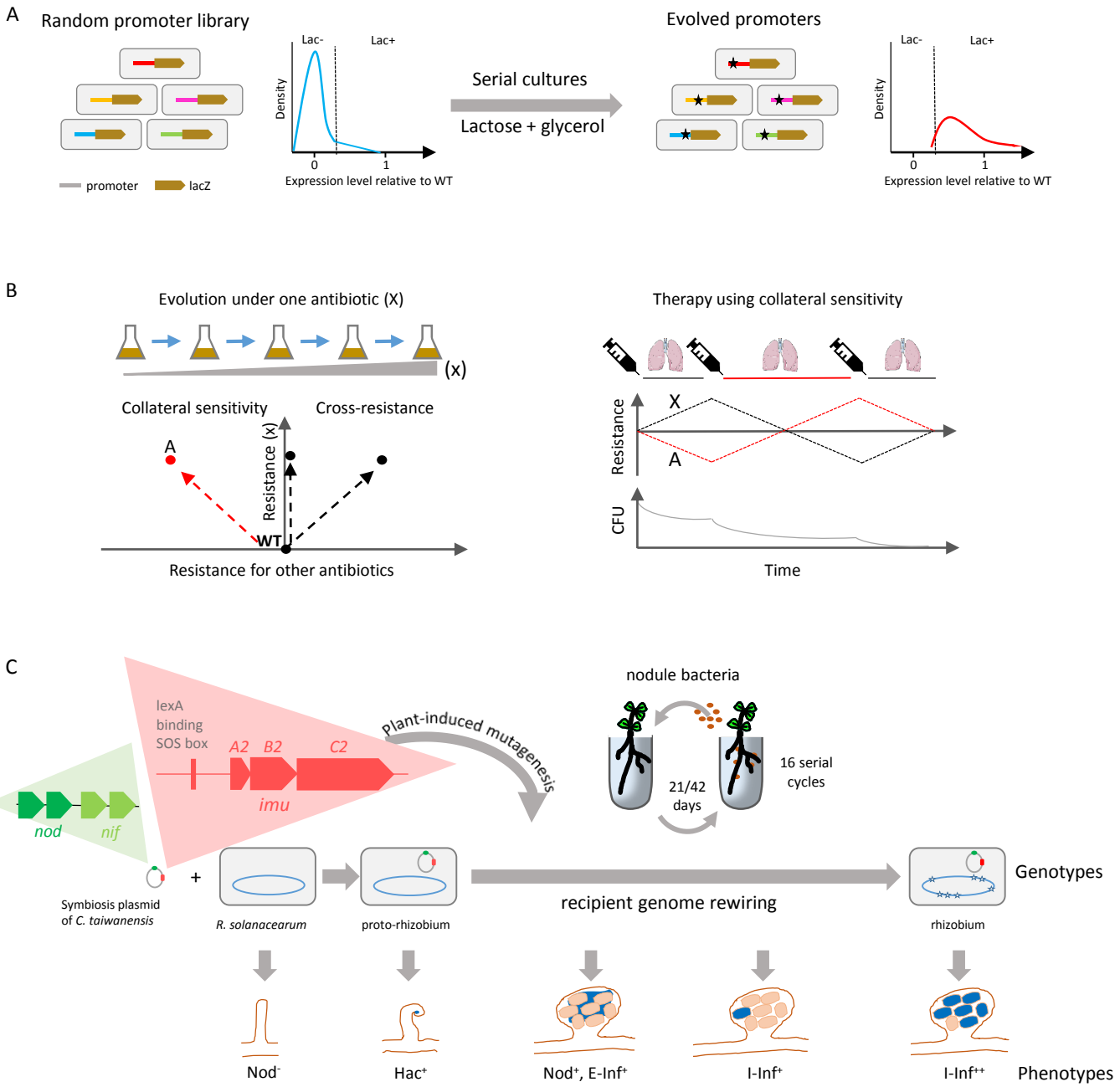
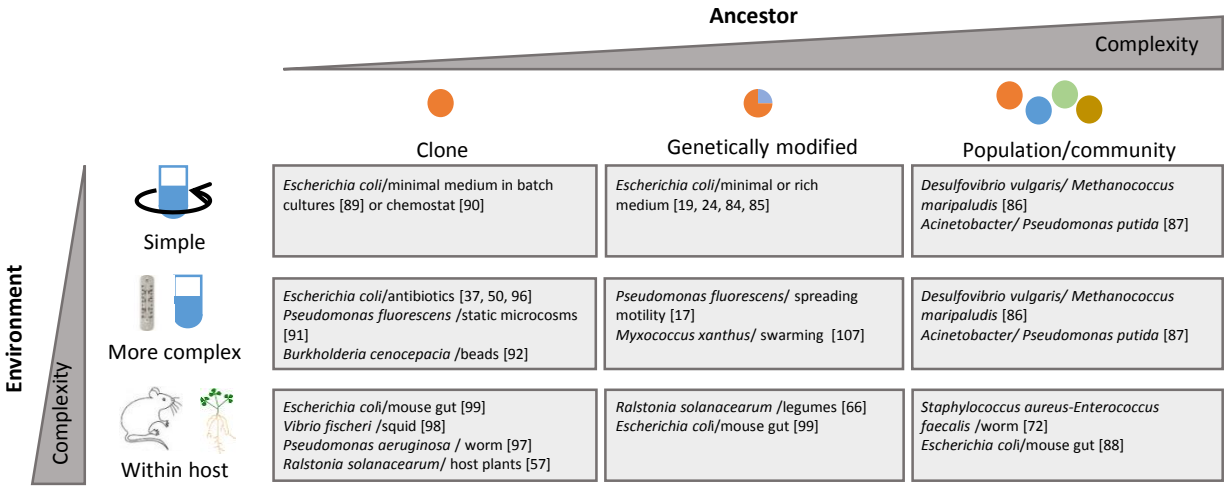


Figure 1 for Box 1 (revised)



Highlights

- Experimental evolution (EE) can complement traditional molecular genetic studies on microbial systems.
- The diversity of EE approaches enabled progresses 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.

Outstanding questions:

- Can we exploit mutations found in EE to characterise genes of unknown function?
- Can EE enlighten mechanisms slowing down the evolution of resistances?
- 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, obligate endosymbionts of insects)?