NATURE|Vol 457|12 February 2009|doi:10.1038/nature07892

The *Beagle* in a bottle

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Why infer evolution when you can watch it happen in real time? This is the basic premise of using populations of fast-replicating microorganisms in test tubes to study evolution. The approach, known as experimental evolution, has provided a way of testing many of the key hypotheses that arose from the modern evolutionary synthesis. However, details of the unnatural histories of microorganisms in test tubes can be extrapolated only so far. Potential future directions for the approach include studying microbial evolution for its own sake under the most natural conditions possible in the test tube, and testing some qualitative theories of genome evolution.

Charles Darwin's insights into the nature and mechanism of the evolutionary process were built on careful observation of the patterns of variation across the natural world. In *On the Origin of Species*[1], Darwin brought together evidence from an incredible diversity of taxa to support his case. But one major group notably lacking from this work, the microorganisms, has since provided some of the best experimental support for the theory of evolution by natural selection[2]. In this Review, we examine how microorganisms, and in particular evolution experiments using microorganisms, have helped to unlock the details of the adaptive process. In particular, we highlight some of the most important insights that this approach has provided, those that we think would have excited Darwin, and we discuss the important role that this approach will continue to have in furthering the understanding of organic diversity.

When Darwin developed his theory of natural selection, he assumed that evolutionary change would usually be slow and, in general, not directly observable. As he wrote in *On the Origin of Species*: "We see nothing of these slow changes in progress, until the hand of time has marked the long lapses of ages, and then so imperfect is our view into the long past geological ages that we only see that the forms of life are now different from what they were."

The fossil record was valuable in supporting his thesis, but it was also imperfect and open to multiple interpretations. However, one of Darwin's crucial realizations was that the process of natural selection had an analogue in the artificial selection applied by breeders of animals and plants. Darwin frequently used examples from fields such as pigeon breeding to illustrate the power of cumulative selection to generate rapid change in biological organisms. The value of such indirect evidence into the power of selection is indisputable, but how much more powerful is it to observe natural selection directly and in real time?

What is experimental evolution?

Experimental evolution has transformed evolutionary biology from a historical science, in which unseen processes are inferred from evolutionary end points, to one in which evolution can be studied in real time. The method involves propagating replicate populations of living things in defined laboratory environments for tens, hundreds or even tens of thousands of generations. The experimenter controls the environmental conditions but does not directly impose the selection, as is the case with pigeon breeding. Instead, selection results from the "struggle for existence" between individuals within each population; in other words, selection is natural. A wide range of organisms have been studied in this way, including nematodes, fruitflies and mice[2], but by far the easiest and most productive organisms to work with are microorganisms, primarily bacteria, yeast and viruses. Their large populations and short generation times favour rapid evolution, and the ability to store populations cryogenically in suspended animation allows direct comparisons to be made between evolved, ancestral and intermediate forms, providing researchers with a 'living fossil record'[3]. Moreover, many microorganisms have relatively simple and well understood genomes, allowing both genetic manipulation and, to some extent, identification of the genetic targets for selection.

Perhaps the first experimental evolutionist was William Dallinger, a contemporary of Darwin. Using equipment not dissimilar to that seen in modern-day microbiology laboratories, Dallinger maintained populations of flagellates under conditions of gradually increasing temperature[4]. After several thousand generations, his flagellates had evolved to grow at temperatures far exceeding the thermal range of their ancestors, providing a dramatic demonstration of the power of natural selection to adapt organisms to changing environmental conditions. It is an interesting historical note that Darwin, although fully aware and supportive of this work, does not seem to have appreciated its value as evidence in support of his theory.

Despite this long history, the field of microbial experimental evolution did not take off until the early 1990s, following the publication of work by Richard Lenski and colleagues[3] on the long-term adaptation of the bacterium *Escherichia coli* to a novel laboratory environment. This experiment contains many elements common to experimental evolution studies and is outlined in Box 1. This work led to a rapid rise in the popularity of microbial experimental evolution as a method for studying evolution (Fig. 1), and it has been used to address questions spanning the entire spectrum of the modern evolutionary synthesis, from the tempo and mode of adaptation and diversification to the evolution of sex and sociality.

What has been learned

Experimental evolution has provided a wealth of information about the way evolution works by allowing researchers to test their theories directly. Here we consider some of the key findings that sprang from this work.

The importance of chance in the adaptive process

Darwin proposed that natural selection would lead to adaptation and organisms that are well fitted to their environment. The incorporation of genetics into Darwin's evolutionary framework (the 'modern evolutionary synthesis')[5] led to a number of explicit predictions concerning the dynamics of adaptation, many of which have now been confirmed by

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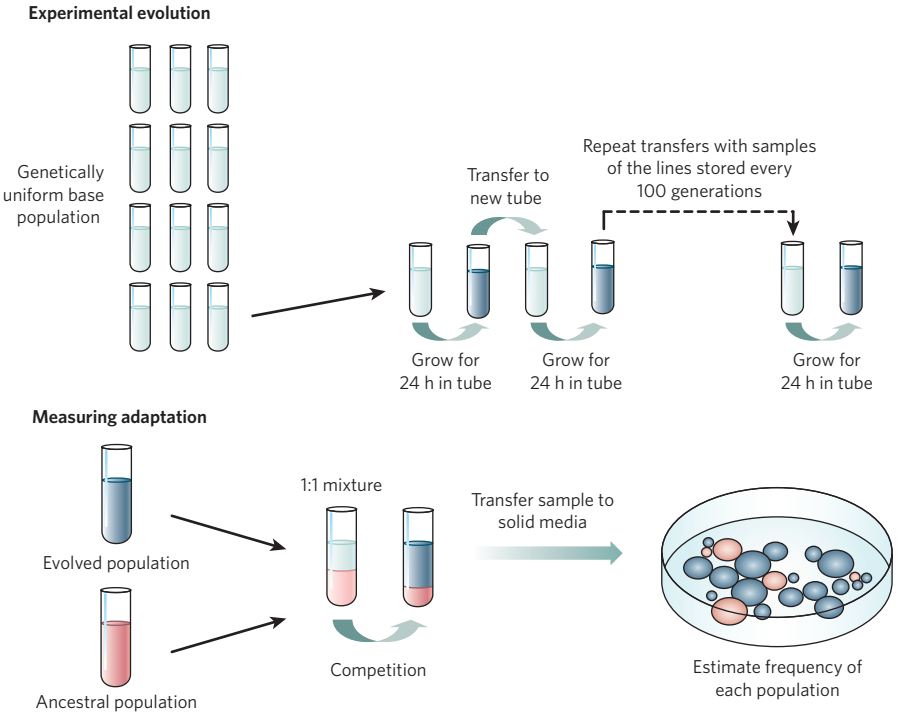
Box 1 | The long-term selection experiment



On 15 February 1988, Richard Lenski started a selection experiment that continues to this day. The experimental design is startlingly simple: 12 populations of the bacterium *Escherichia coli* B were grown in identical flasks containing a simple liquid medium with glucose as the sole carbon source. Every day, 1% of each population was transferred to a fresh flask (see box figure, top). For *E. coli* B, this was a new environment, and one to which it was initially poorly adapted. Each 24-hour growth cycle allowed 6.6 bacterial generations, and after every 100 generations a sample of each population was stored in the freezer alongside the ancestral *E. coli* B strain. Because the 12 populations were initially genetically identical, the only source of variation for natural selection to act on was *de novo* mutation.

The adaptation of each population to the environment was assessed by directly comparing the evolved bacteria to the *E. coli* B ancestor in head-to-head competition experiments under exactly the same conditions as one 24-hour growth cycle of the selection experiment (see box figure, bottom). Samples of the evolved and ancestral populations (the latter with a genetic colour marker) were removed from the freezer and grown individually. Small numbers of bacteria from both populations were then mixed in a 1:1 ratio in the same tube and allowed to compete for 24 hours. Samples of the mixture were then grown on solid media that allow colonies derived from evolved and ancestral cells to be distinguished on the basis of their colour. The change in the ratio of the types was then used to estimate the fitness of the evolved lines relative to the ancestor.

In 1994, the results of 10,000 generations of the selection experiment were published[7]. This revealed that the relative fitness of the 12 experimental populations had increased by approximately 40% but that most of this increase occurred in the first 2,000 generations of evolution. Cell size almost doubled during 10,000 generations, following a similar trajectory to relative fitness, with the most rapid increase over the first 2,000 generations. Importantly, the evolutionary trajectories of both relative fitness and cell size, and the relationship between them, were subtly different between the replicate populations, suggesting that chance and contingency, as well as natural selection, had important roles in the evolution of these populations.

experimental evolution. One particularly robust example is the way the rate of adaptation changes through time: fitness initially increases rapidly as mutations that have a large effect become fixed, and then plateaus as beneficial mutations become rarer and have a smaller effect[6]. One of the reasons for this consistency between theory and experiments is the large population sizes of microorganisms studied, which increases the predictability of the evolutionary process. However, chance does, surprisingly, still have an important effect, even in massive populations. For example, initially identical populations of *E. coli* placed in identical, simple environments follow different evolutionary trajectories, in terms of both fitness itself and, even more strikingly, in phenotypic traits such as cell size[7] (Box 1). Genetic analyses of these populations have shown that, by chance, different random mutations, albeit often in the same gene, have been fixed in different populations, subtly altering the trajectory of evolution[8,9]. The fact that subsequent mutations were then contingent on prior mutations (epistasis) acted to reinforce this divergence[10].

The ability to evolve may itself evolve

Natural selection operates on genetic diversity, so it follows that mechanisms that generate diversity may themselves be under selection. Indeed, Darwin recognized that sexual reproduction may be an example of an evolvability mechanism[1], as it allows more rapid adaptation of populations. Because the benefits of sex can take considerable time to be realized in multicellular organisms, facultatively sexual microorganisms (such as yeast[11] and single-celled algae of the genus *Chlamydomonas*[12]), provide a unique opportunity to determine the conditions under which sex is advantageous. Whether the organisms have sex can be manipulated by the experimenter in a variety of ways, and otherwise identical sexual and asexual populations are allowed to evolve under defined conditions. The main conclusion from these studies is that sex does indeed favour more rapid adaptation but only when populations are both large and poorly adapted to their environment[11,12]. In most cases, the key benefit of sex is that it brings together beneficial mutations in the same individual that would otherwise be competing against each other in different individuals, an idea originally proposed in the 1930s[13,14].

A more fundamental mechanism of evolvability is mutation itself. In a well-adapted organism, most mutations are likely to be deleterious, so it might be expected that natural selection drives mutation rates down until the physiological costs of error correction become too great[15]. However, for asexual microorganisms, increased mutation rates, which generally result from dysfunctional DNA proof-reading enzymes[16], may confer an indirect advantage by generating a larger number of beneficial mutations that

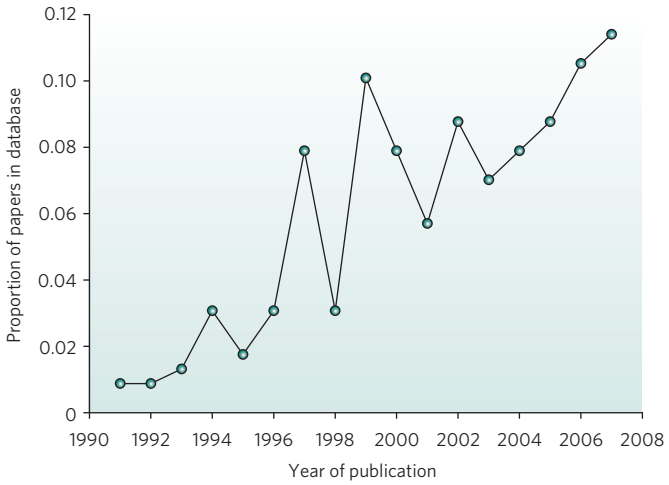


Figure 1 | The rise of experimental evolution. We constructed a bibliographical database of experimental evolution papers by searching the ISI Web of Knowledge for papers that contain the keywords 'experimental evolution' or 'selection experiment' and were published between 1991 and 2007. This search was then manually edited to produce a database of experimental evolution papers involving microorganisms. The reduced database contains 268 papers, which is an underestimate of the total size of the literature in the field, but we have no compelling reason to think that this database is not a random sample of the experimental evolution literature. The graph shows the proportion of the papers in this reduced database that were published in each year.

effectively offset the costs of the deleterious ones[17]. Crucially, bacteria with increased mutation rates ('mutators') have been observed to evolve *de novo* in the laboratory[18–20]. As with sexual reproduction, increased mutation rates are typically favoured in populations that are poorly adapted to their environment. However, unlike sex, increased mutation rates are more beneficial in small populations that would otherwise spend longer than large populations waiting for the next beneficial mutation[21].

Diversity is the rule and not the exception

It was the great diversity of life on Earth that inspired Darwin to develop his theory of natural selection, and the question of why there are so many species continues to fascinate evolutionists to this day. In an early example of experimental ecology, Georgii Gause's studies[22] on coexistence of competing species of the unicellular ciliate *Paramecium* gave rise to the principle of competitive exclusion, which states that diversity cannot be maintained without environmental complexity in the form of multiple ecological niches. Experimental evolution has extended this finding over evolutionary timescales, showing that the amount of diversity that evolves from initially genetically uniform populations increases as a function of environmental complexity, in terms of spatial heterogeneity[23] (Fig. 2) or types of food resource[24]. Furthermore, these studies show that fitness trade-offs between different ecological niches readily evolve and are crucial if diversity is to be stably maintained. However, what is perhaps more surprising is that stable diversity, and hence specialization, can also evolve in apparently homogeneous environments containing a single food resource[25–29]. This diversity results from trade-offs between primary resource and waste metabolite utilization[27], but more fundamental biochemical trade-offs between the rate and yield of energy production are also likely to be important[30]. The main conclusion of these studies is that environmental complexity influences diversity, but given enough time and competition for resources, diversity is always likely to evolve.

Costly social action can be explained by kin selection

Like all organisms, microorganisms have social lives, with individual cells carrying out a range of extracellular actions that can affect the fitness of nearby cells[31]. These include the formation of multicellular structures and the production of enzymes and chelators to release nutrients from the environment[31,32]. However, many of these social actions are metabolically costly to individual cells yet provide a benefit to the group, and as such are open to exploitation by social 'cheats' who reap the rewards of social action without paying the costs. In *The Descent of Man*[33], Darwin suggested that individually costly social action could evolve if the benefits accrued to family members, an evolutionary process later formalized by W. D. Hamilton as kin selection[34]. By manipulating the genetic relatedness of groups of interacting cells, evolution experiments using a wide range of microorganisms and their social traits have shown that cooperation only persists when genetic relatedness is high[31]. For example, the opportunistic pathogen *Pseudomonas aeruginosa*, like most bacteria, produces extracellular iron-scavenging molecules, known as siderophores. Social cheats that produce fewer siderophores but can still use the iron-bound siderophores produced by others readily evolve and increase in frequency from wild-type populations[35] (Fig. 3). High siderophore production can be maintained only when populations are exposed to strong genetic bottlenecks, ensuring that relatedness is high[36]. Moreover, this study[36] also confirmed theoretical predictions that competition between groups (allowing productive groups to produce more propagules) is crucial for the evolution of costly cooperation. There are numerous other examples of the role of kin selection in explaining cooperation in microorganisms, most notably that of social amoebae (such as slime moulds of the genus *Dictyostelium*) sacrificing themselves to form stalks that support viable spores[37].

Species co-evolve

Darwin recognized the importance of co-evolution, which is the reciprocal evolution of species or populations in response to each other. He saw it in particular as an explanation for the good fit of the length of pollinators' tongues to the nectar spurs of the flowers they visit[1]. Co-evolution is likely to be a powerful force because a change in one species alters selection on the other species at a much faster rate than that typically caused by changes in the physical environment[38]. The most extreme example of rapidly changing selective pressures is that resulting from the interaction between hosts and their lethal parasites. The evolution of parasite-resistant hosts from a previously sensitive population will drastically change the parasite's lot from utopian to the brink of extinction, and likewise for the host population if the parasites evolve to overcome this resistance. Because of the potential speed of host–parasite co-evolution, it has been implicated as a key force in a range of evolutionary processes, including speciation[38] and the evolution of sex[39]. Yet it has proven to be one of the most elusive of evolutionary phenomena to detect in natural populations. Studies using microorganisms, primarily bacterial hosts and parasitic viruses (Fig. 4), have allowed co-evolution to be directly observed by taking advantage of the living fossil record[40–43]. Populations can be assayed against past, contemporary and even future enemy populations, allowing the reciprocal evolution of resistance and infectivity to be directly measured. These studies not only have confirmed the rapidity and persistence of co-evolutionary change but also have allowed tests of the consequences of co-evolution. For example, co-evolution can radically alter host genetic diversity[44] and impose selection for evolvability (increased mutation rates)[20]. Experimental studies that focus on the evolution of mutualisms (specifically, in *E. coli* and plasmids that carry antibiotic resistance genes) have shown that such mutualistic interactions become more and more specialized over evolutionary time[45], confirming Darwin's hunch.

Criticisms and caveats

Despite the success of microbial experimental evolution in providing crucial tests of evolutionary theory, there are inevitably limitations. In this section, we examine five of the most common criticisms.

Test tubes are unnatural environments

It may be difficult to show how evolution works in nature by studying it in an unnatural environment, such as a test tube. However, although a test tube is unnatural at the beginning of the study, after the hundreds or thousands of generations in a typical study, it becomes a natural environment with respect to the microorganism's recent evolutionary history[3]. By comparison, many of the organisms so beloved of researchers investigating

evolution in the wild are relative newcomers to their current environments, as is the case for the well-studied Soay sheep population that was introduced to the Scottish island of St Kilda about 4,000 years ago with human settlers[46]. Thus, a great advantage of evolution in test tubes is that the organisms have evolved in precisely the same environment in which they are subsequently assayed. Compared with taking organisms out of their environment and measuring them under laboratory conditions, the advantages of this approach are clear.

Test tubes are too simple to examine real-world complexity

The criticism that a test tube cannot mirror the complexity of natural environments misses a key rationale behind this approach. Experimental evolution does not seek to mimic specific systems in all their complexity. Instead, the microcosms are biological models in which researchers attempt to capture the essence of evolving systems in general and so can shed light on general processes that are expected to occur in all life on Earth. Evolutionary biologists have successfully used simple mathematical models to provide insight into fundamental evolutionary phenomena. No one would argue, for example, that because R. A. Fisher's fundamental theorem[13] does not contain parameters for every aspect of the biology of even a single species, it can suggest nothing useful about evolution. Simplicity is a strength, rather than a weakness. Experimental evolution can provide support for a given theory, but it indicates little about its relative importance in explaining patterns in nature where many selective forces are likely to act simultaneously. Experiments can be made more complex, with many interacting selective forces included[47], to provide a clearer picture of the ecological and genetic mechanisms studied. Paradoxically, however, such complexity may not be the most productive use of experimental evolution because, by definition, generality is lost. Again, the analogy with theoretical models is clear: the more parameters a model has, the less generally useful it becomes.

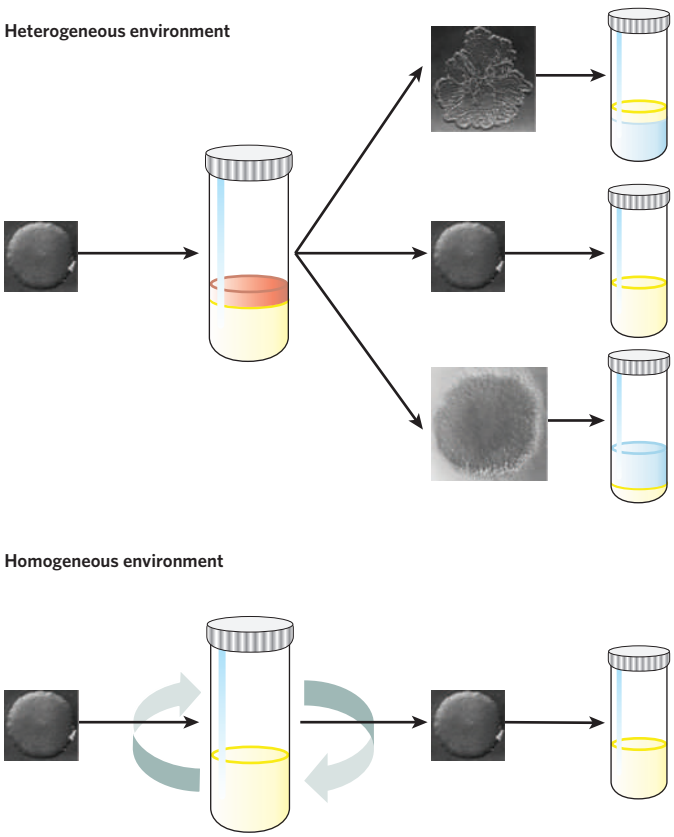


Figure 2 | Adaptive radiation in a heterogeneous environment. Paul Rainey and Mike Travisano placed a single clone of the bacterium *Pseudomonas fluorescens* into two different environments, one with and one without spatial heterogeneity[23]. After several days, the bacteria grown in each environment were plated onto solid medium. The colony grown in the presence of spatial heterogeneity gave rise to a diverse range of colony morphologies, whereas the colony grown in its absence did not. Crucially, these different colony morphotypes were each adapted to occupy a different region of the liquid broth (right; yellow band). This finding was tested by re-inoculating each morphotype into a fresh sterile microcosm of liquid broth. (Images reproduced, with permission, from ref. 23.)

Microorganisms are different from larger organisms

Certain questions are clearly beyond the reach of experimental evolution using microorganisms, such as the evolution of consciousness. However, biologists unfamiliar with microorganisms may be surprised at how similar the lives of microbiota and macrobiota are. As discussed earlier, some microorganisms have sex (although, to complicate matters, bacteria and viruses transfer genes between species[48]), and all microorganisms have complex social lives, in which they communicate, cooperate and are spiteful to one another[31]. It has recently been shown that they even grow old and decrepit: the poles of bacteria are subject to ageing[49,50], allowing experimental tests of the hypothesized trade-off between reproduction and ageing[51]. The key is to choose an organism that fits the question, as is the case in all areas of evolutionary ecology.

Microevolution cannot be scaled up

Several experimental evolution studies have framed their results in the context of species-level, or macroevolutionary, change. For example, the replacement of one mutation by another, followed by a period of evolutionary stasis, has been interpreted as supporting a punctuated-equilibria model of macroevolution[52], and the rapid diversification of a single bacterial clone into genetically distinct niche specialists has been described as adaptive radiation[23]. Changes in a test tube are largely the result of a few point mutations or insertions[8,53], but they have large phenotypic effects as a result of the novelty of the laboratory environment. This is clearly not macroevolution, because genotypes are not species. But similar processes are likely to be driving microevolutionary and macroevolutionary change. For example, adaptive radiations are almost certainly driven by competition and ecological opportunity[54]; exactly the same mechanisms have been identified in evolution experiments. Furthermore, recent experimental evolution studies have detected the signatures of speciation. For example, the theory of ecological speciation suggests that reproductive isolation evolves as a by-product of divergent ecological selection[54], and this has recently been shown in experimental populations of yeast[55]. Perhaps macroevolution could occur in test tubes after all.

Experimental evolution studies are contrived

The control afforded by experimental evolution can lead to rather inevitable outcomes. This is especially so when experiments are carried out over short timescales using genotypes whose phenotypic characteristics have already been defined in the selective environment. For example, several studies have begun with mixtures of two genotypes, a wild type and an isogenic mutant with a particular gene knocked out, and followed the frequencies of these genotypes for a few tens of generations. Such studies can provide a useful biological demonstration of theory and may be the only feasible way to quantify weak selective forces, but the true strength of these studies is that experiments can be carried out over timescales in which the evolutionary outcomes are not limited by the genetic variation initially present. Such studies allow researchers to examine processes that occur beyond the immediate short term and that are not easily examined in other ways.

The future

We have discussed how experimental evolution studies have provided insights into phenotypic evolution in real time and the genetic changes underlying it. But what might such studies show about the evolution of genomes themselves? And can they be applied to solve problems in the real world?

Experimental evolution at a genomic scale

The specimens catalogued so carefully by Victorian naturalists, which inspired Darwin's ideas, have been replaced by a new kind of specimen:

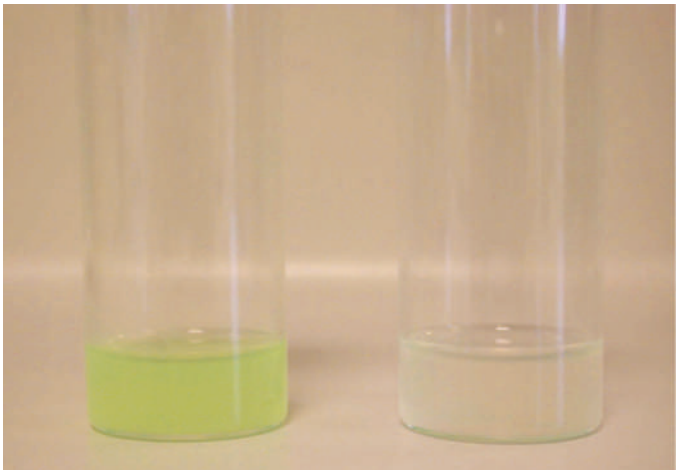


Figure 3 | Cooperation and cheating in *Pseudomonas aeruginosa*. The tube on the left shows a wild-type cooperating population of the bacterium *P. aeruginosa* producing pyoverdine, an extracellular, iron-scavenging molecule (or siderophore) that is a yellow-green colour. The tube on the right shows a pyoverdine-negative 'cheat', which can exploit the siderophore but does not produce it.

the genome sequence. The ability to sequence genomes, and measure transcriptomes and proteomes, increases almost daily, and computational developments in metabolic-network analysis are increasingly resulting in a change away from simple observation towards hypothesis-driven questions[56–58]. What determines the physical order of genes in the genome? When do genomes evolve to be robust to mutation? How do networks of genetic regulation evolve? What is the minimal genome for a particular set of environmental conditions? Fortunately, the microorganisms used in experimental evolution studies are cheap and easy to sequence, and it is increasingly likely that it will be possible to carry out large-scale sequencing of not only the end points of selection experiments but also of many individuals in between. Experimental evolution has, in the past, allowed evolution to be observed directly at the phenotypic level; in the future, it will provide a means of examining directly the processes that mould the evolution of genomes themselves. Some of the earlier studies of experimental evolution set out to address these types of question[59], but the technological advances that accompanied the genomic revolution will allow researchers to address them in a much more systematic way.

Despite the incredible potential of experimental evolution as a tool to study the evolution of genomes, we can see significant challenges to the development of this field. Experimental evolution studies succeed when they explicitly test clearly defined and general hypotheses that should apply to any biological system, and the success of 'omic'-scale studies in experimental evolution will depend on their ability to stay within this hypothesis-driven framework. Many experimental evolution studies that investigated the genetic basis of adaptation had the goal of simply identifying the mutations underlying adaptation to a particular laboratory environment. However, the arbitrary and artificial nature of selective environments imposed by researchers makes it questionable how relevant this approach is for understanding evolution. Another challenge will be to understand the functional consequences of genetic variation: producing long lists of mutations that are responsible for adaptation could amount to little more than 'stamp collecting' unless the consequences of mutations are adequately characterized.

Finally, many of the most spectacular examples of adaptation in selection experiments result from modest changes at the genomic level[60–62], suggesting that experiments designed to investigate broad features of genome evolution will need to find ways to accelerate adaptation or find new model systems to work with. Experiments involving thousands of generations might not be sufficient to understand the underlying causes of some of the differences in genome structure and content that are clear from comparing the genomes of species that have diverged from each other over millions of years.

Studying microorganisms for their own sake

Experimental evolution studies using microorganisms are dominated by tests of universal theories, but the approach can also improve knowledge of the evolution and ecology of the microorganisms themselves. This is clearly a useful aim, given the ubiquity of microorganisms and their important role in all ecosystems. However, it is crucial to use nature as a guide. It is most useful to identify phenotypes that are clearly under selection in natural populations, as well as the key selective forces that operate on them. For example, public-goods cheats in the bacterium *P. aeruginosa*[63,64], which produce less siderophore than normal and are often associated with reduced virulence[65,66], are much more prevalent in chronic infections than in the initial colonizing populations[63,64]. Compared with success in the external environment, success within hosts imposes considerably different selective pressures (such as immunity, predation, antibiotics and scale of competition), and experimental evolution studies can determine the relative importance of these forces in creating natural patterns of social behaviour. Using experimental evolution to determine how microorganisms respond to selective pressures in their natural environments will also allow their evolution to be anticipated. This is likely to be particularly useful for testing the propensity to evolve resistance to antibiotics[67].

The next logical step in studying the evolution of microorganisms is to carry out experimental evolution in natural environments. At first this may seem to disregard one of the key advantages of the whole approach: control over the environment. However, 'natural microcosms', such as tubes of soil or pond water, or even isogenic hosts[18], can be replicated, and environmental and genetic variables manipulated. The crucial difference between such studies and traditional experimental evolution is that the importance of the manipulated selective pressure can be determined among all the other natural selective pressures.

Evolving helpful microorganisms

Humans have used selection in a range of organisms, including microorganisms. For example, the lactic-acid-producing bacterium *Streptococcus thermophilus*, which is used in the production of yoghurt, has been artificially selected for thousands of generations, possibly from a human oral commensal ancestor[68], and brewing and bread yeasts have been artificially selected from naturally occurring grape yeasts. Despite this illustrious history of applied selection experiments on microorganisms, selection is not being fully exploited to produce microorganisms that will be useful in medical, environmental or agricultural contexts. For example, bioreactors, which rely on microorganisms to break down or produce chemicals, sometimes fail[69] because of colonization by more competitive

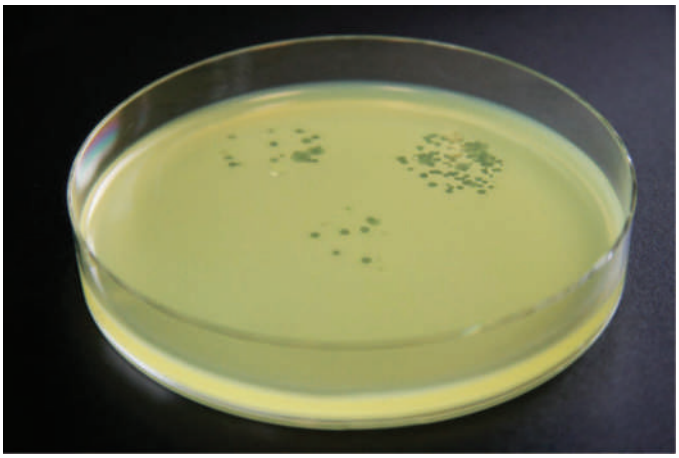


Figure 4 | Experimental assay to show bacteriophage infecting bacteria. Each plaque, which contains approximately 10^5 bacteriophage particles, indicates that a single phage particle has successfully established an infection in a lawn of *Pseudomonas fluorescens* bacteria. This assay provides a simple means to follow the co-evolution of bacterial resistance and bacteriophage infectivity.

microorganisms from the environment. A great advantage of experimental evolution is that it is driven by natural selection, so the traits required can be artificially selected at the level of the population, and the fittest organisms, and crucially those most resilient to competition from foreign invaders, will dominate. Similarly, experimental evolution can be used to evolve biological control agents, such as highly evolvable bacteriophages that could be used to treat antibiotic-resistant bacterial infections[70].

Given these exciting future prospects, we anticipate that experimental evolution using microorganisms will expand at an even faster rate than in recent years. Darwin would no doubt have been delighted by the insights that experimental evolution has provided into understanding and applying his big idea: evolution by natural selection. ■

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Acknowledgements We thank the Royal Society and the Leverhulme Trust for funding.

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