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Seeing is believing: what experiments with microbes reveal about evolution

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Darwin's theory of natural selection is among the most powerful ideas in science, yet evolutionary ideas remain challenged to this day. This is in part because evolution often cannot be directly observed. Simple experiments with microbes can change that by enabling direct observation of evolutionary processes.

Evolution in a Petri dish

We recently carried out an evolutionary experiment where we made the bacterium *Pseudomonas aeruginosa* go through consecutive rounds of swarming. Swarming is a form of collective motility where *P. aeruginosa* colonies travel over soft surfaces in a branched pattern. Over the course of this experiment the colonies evolved a new morphology called hyperswarming where they cover the entire plate rather than branch out. The genome of *P. aeruginosa* has 6 million nucleotides and finding the causative mutations, which is like finding a needle in a haystack, could have been very costly even 5 years ago. These days, next generation sequencing and bioinformatics methods are more accessible and it was relatively easy to find the mutations. We used classical approaches, gene complementation, and transcription expression assays to confirm the sequencing results and understand the molecular mechanisms involved. Hyperswarming was caused by single point mutations in the gene *fleN*, which turned the normally monoflagellated *P. aeruginosa* into a polar multiflagellate and improved swarming [1].

These results shed new light on the genetics of flagella. Before our experiment it was known that *fleN* regulates flagellar number but *fleN* knockout mutants cannot swim, so finding hyperswarmer mutations in this gene was unexpected. Furthermore, the same mutations that enabled the bacteria to swarm better also impaired their ability to form biofilms, a finding that can potentially be exploited in anti-biofilm therapies [2].

In addition to molecular insights, the hyperswarming experiment has two features relevant for evolution. First, the experiment is surprisingly reproducible. All hyperswarmers evolved in independent experiments had point mutations in *fleN*. This was difficult to anticipate because swarming in *P. aeruginosa* involves many genes [3] and our finding supports that evolution can be, under certain conditions, predictable. Second, the mutations produce changes at the cellular level that lead to better colony spreading. Hyperswarming is easy to explain in pictures just like a textbook example of experimental evolution (Figure 1).

Our study is, of course, not the first to adapt microbiology assays for experimental evolution (see a recent review [4]). Microbes make great models owing to short generation times and huge population numbers, and evolution can itself be used as a tool for microbial genetics [5]. The same integration of molecular and evolutionary methods is within reach for every experimental microbiologist and can lead to powerful demonstrations of evolution. Simple experimental evolution with harmless bacteria, such as the non-pathogenic relative of *P. aeruginosa*, *Pseudomonas fluorescens* [6], can even be adapted for educational purposes [7].

In order to do experimental evolution it helps to consider a straightforward definition: evolution is the change of gene frequency in a gene pool. The definition derives from the work of Fisher, Haldane, and Wright, who integrated Mendelian heredity and Darwin's natural selection into population genetics [8]. Gene frequencies can change through four mechanisms: mutation, natural selection, genetic drift, and migration. Mutation and natural selection readily come to mind when thinking about evolution. Mutation, a change in the heritable material, is a process that generates genetic diversity. Selection acts on that diversity, favoring alleles with increased reproductive success. Drift and migration may be less discussed but are equally important. We present examples of all four processes below. Any evolutionary experiment can be interpreted according to these four elementary mechanisms, as long as the gene pool and a timespan of evolution are properly defined. Experiments are often a combination of some or all of these four processes. Here are some notable examples.

Mutation and selection

In 1943, Luria and Delbrück published their famous fluctuation experiment [9]. They asked whether the resistance to bacteriophage in *Escherichia coli* was induced by the presence of phage or if instead it was due to random mutations occurring prior to phage exposure. Plating *E. coli* on agar plates with phage and counting colonies followed by sound theoretical analysis led to the conclusion that resistance came from prior mutations. This was the first direct demonstration of the random nature of mutation [10]. Luria

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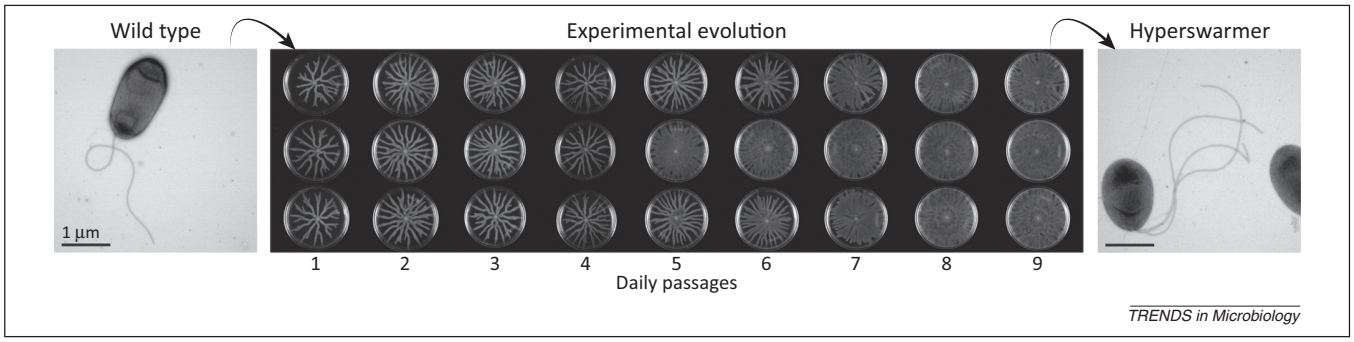

Figure 1. The hyperswarming experiment. Three independent lineages were started from the same ancestral *Pseudomonas aeruginosa* (left panel) and passaged daily in swarming assays in Petri dishes [1]. Over the 9-day experiment, the colony morphology evolved from a branched pattern to a round shape covering most of the Petri dish, a phenotype we call hyperswarming (center panel). In replicate experiments, hyperswarming was always caused by point mutations in the same gene, the flagellar synthesis regulator *fleN*, which make the bacteria change from being monoflagellated to being multiflagellated (right panel).

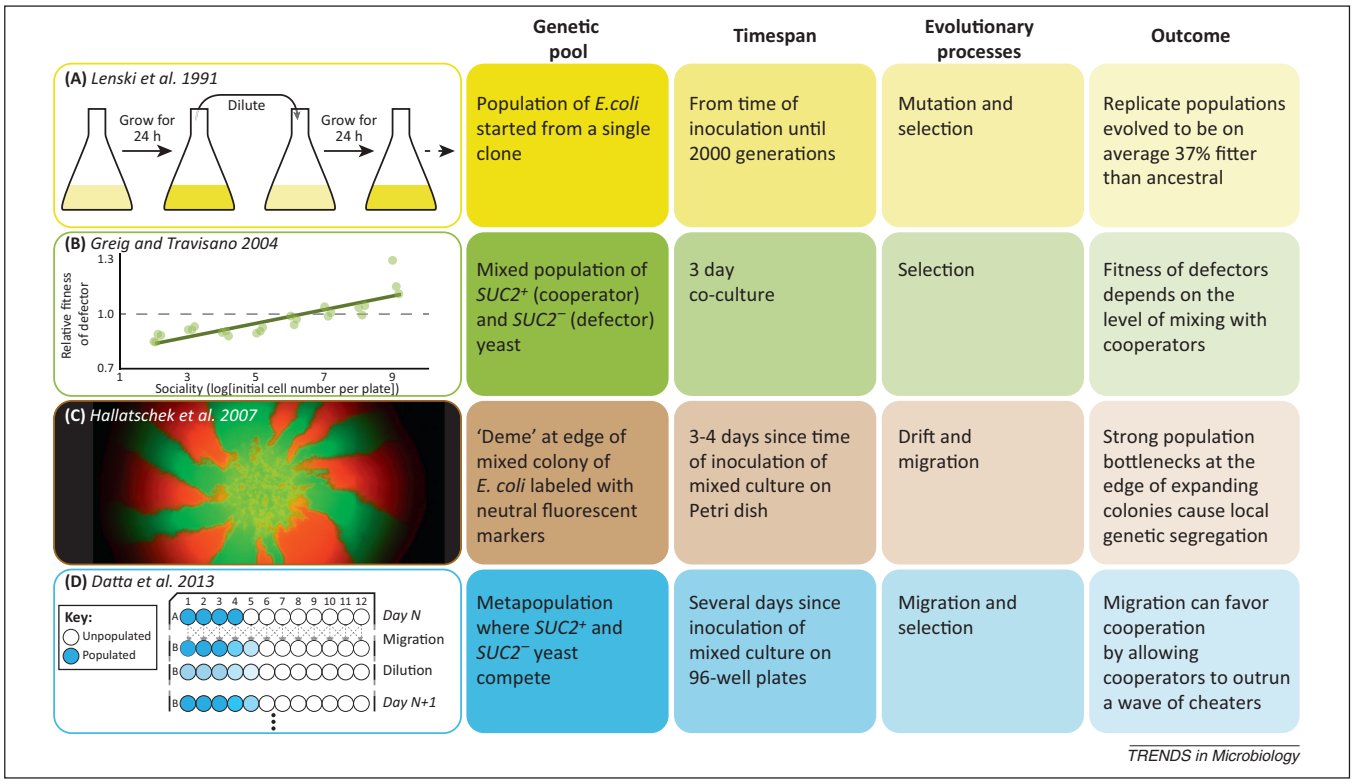

Figure 2. Microbial models of evolution. **(A)** The long-term evolutionary experiment of Lenski and colleagues consists of the daily passaging of parallel populations of *Escherichia coli* [11]. **(B)** Investigation of frequency-dependent selection by mixing two strains and measuring their numbers before and after competitions. Plot adapted from the experiment of Greig and Travisano using the yeast *SUC2* model [13]. **(C)** Genetic drift is observed in bacterial colonies by mixing two variants of *E. coli* differing only in the expression of a fluorescent marker (shown in green or red) [14]. Picture courtesy of Oskar Hallatschek. **(D)** An experimental analog of the 'stepping-stone' model used to study the role of migration in the evolution of cooperative yeast [15].

later was the PhD advisor of Watson who, with Crick, identified the structure of the heritable material, DNA. The work of Luria and Delbrück launched the discipline of molecular biology and they received the Nobel Prize in 1969.

In 1991, Richard Lenski and colleagues published the first results on a long-term experimental evolution [11]. The experiment consisted of daily passages of 12 populations of *E. coli* started from a single clonal ancestor (Figure 2A). The populations evolved over 2000 generations, becoming on average 37% fitter. Consistent with the randomness of mutations, the populations evolved at different rates. The long-term experiment of Lenski has now been running for more than 25 years and is rapidly approaching 60 000 *E. coli* generations. It has inspired many studies, of which the hyperswarming experiment is but one example [1].

Frequency-dependent selection

In some cases, the fitness of an organism depends on what other organisms in the same population are doing. For example, organisms that carry out cooperative actions face a problem when others in the same population are not cooperative. If selection favors selfishness how can cooperators resist exploitation? How cooperative behaviors evolve is a fundamental issue in evolution [12] that has become tractable through microbial experiments. *Saccharomyces cerevisiae* requires the gene *SUC2* to break down

sucrose into fructose and glucose. Strains lacking *SUC2* cannot grow on sucrose. In a co-culture experiment $SUC2^-$ mutants exploit $SUC2^+$ strains because they can metabolize free glucose and fructose without contributing to sucrose digestion. Thus, selection for *SUC2* is frequency-dependent (Figure 2B), which may explain why the SUC family of genes is polymorphic [13].

Drift and migration

Although genetic drift is evolutionarily important, it is not necessarily intuitive. Fortunately, drift can be observed in simple experiments using neutrally labeled *E. coli* strains. When growing a two-color mix of *E. coli* spotted on a Petri dish for 3–4 days, it will look normal under regular light. Fluorescence imaging reveals unmixing of the two strains in beautiful radial sectors (Figure 2C). The explanation for sectoring is that as the colony grows, the center becomes nutrient-depleted and only bacteria at the edge can reproduce. A stochastic process determines which bacteria make it to the next generation [14]. The sectors emerge from genetic drift, a random change in local gene frequency.

Migration and selection

Most gene pools are not closed systems and gene frequency can change because of migration into and out of the system. Sometimes migration can lead to a paradoxical selection for deleterious mutations. Here, too, microbial experiments can provide powerful insights; 96-well microtiter plates can be turned into analogs of the 'stepping stone' model, a classical model to describe dynamics of subdivided populations in population genetics, and used to investigate how migration shapes evolution (Figure 2D). Again using the yeast *SUC2* system, it was recently shown that migration can favor cooperation in an expanding population through the deterministic enrichment of cooperators at the expansion front, and by allowing cooperators to outrun an invading wave of cheaters [15].

Concluding remarks

Evolution is key to understanding all life; still, key evolutionary ideas remain challenged to this day. This is in part because the evolutionary process is often not directly observed but, rather, it is inferred by comparing present-day species, fossil records, and, more recently, DNA sequences. Experiments with microbes allow the observation of evolutionary change on short absolute time scales. Evolutionary biologists that study organisms that can only be seen by the naked eye can miss the power of microbial models [10]. Microbiologists who focus on molecular mechanisms have established many assays that can be used both to study and to communicate evolution. With exciting advances in experimental evolution and new technology, such as affordable whole genome sequencing, it is time that evolutionary biologists and microbiologists come together towards a better understanding of evolution.

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