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Abstract

Genome sequencing has revolutionized studies using experimental evolution of microbes because it readily provides comprehensive insight into the genetic bases of adaptation. In this perspective we discuss applications of sequencing-based technologies used to study evolution in microbes, including genomic sequencing of isolated evolved clones and mixed evolved populations, and also on the use of sequencing methods to follow the fate of introduced variations, whether neutral barcodes or variants introduced by genome editing. Collectively, these sequencing-based approaches have vastly advanced the examination of evolution in the lab, as well as begun to synthesize this work with examination of the genetic bases of adaptation and evolutionary dynamics within natural populations.

Keywords	genome sequencing; experimental evolution; adaptation; clonal interference; distribution of fitness effects

Corresponding Author	Christopher Marx
Corresponding Author's Institution	University of Idaho
Order of Authors	Eric Bruger, Christopher Marx
Suggested reviewers	Michael Desai, Dmitri Petrov, Jeffrey Barrick

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Abstract

Genome sequencing has revolutionized studies using experimental evolution of microbes because it readily provides comprehensive insight into the genetic bases of adaptation. In this perspective we discuss applications of sequencing-based technologies used to study evolution in microbes, including genomic sequencing of isolated evolved clones and mixed evolved populations, and also on the use of sequencing methods to follow the fate of introduced variations, whether neutral barcodes or variants introduced by genome editing. Collectively, these sequencing-based approaches have vastly advanced the examination of evolution in the lab, as well as begun to synthesize this work with examination of the genetic bases of adaptation and evolutionary dynamics within natural populations.

Highlights

- Genome sequencing of isolates remains the most straightforward way to determine evolved changes.
- Sequencing of mixed evolved population samples reveals existing variation and its dynamics.
- Active approaches using sequencing to track either neutral, lineage-tracking barcodes or methods to generate variation that can be selected upon have opened the door to novel questions at an unprecedented scale.
- Sequencing approaches have closed the gap between analyses of laboratory-evolved populations and evolution occurring in natural microbial populations.

Experimental evolution before the application of genome sequencing

Studies delving into microbial evolution date back to early experiments involving pond microbes conducted by the reverend William Henry Dallinger in the late 1800s [1-2]. In the second half of the 20th century, pioneered by researchers such as Bruce Levin, Dan Dykhuizen, and colleagues, the use of evolution experiments in the laboratory became increasingly popular [3-8]. The attraction to this approach was the ability to precisely control the selective environment, transfer regime, and initial genotype, thereby seeding replicate populations that could be cryopreserved as a living fossil record. Upon resuscitation, comparisons could then be made through time, between lineages, and across experiments. A tremendous amount was learned about changes in *phenotype* that occur during adaptation, best exemplified by incredibly fruitful series of discoveries from Rich Lenski's long-term evolution experiment (LTEE) with *Escherichia coli* [9-10]. Stepping back from particulars, some commonalities emerged from the LTEE and other similar experiments. Perhaps most

prominently, the rate of adaptation is almost always fastest early in the experiment, and slows as increasing generations accumulate [10,11]. Conversely, other phenomena were found to behave quite differently depending upon the organism and experiment in question, such as whether replicate populations would exhibit parallelism or divergence in phenotypic changes, or in the extent of tradeoffs between fitness in the selective environment versus alternative environments [12]. Unfortunately, there was generally an inability to link these changes in phenotype with mutations that occurred to the *genotype* [9,13].

Although these numerous experimental evolution studies constituted what was then called 'population genetics without the genetics' [14], in the slightly more than a decade since the first application of whole genome sequencing to experimental evolved populations [15] it is hard to imagine anything further from the truth. Genome sequencing and other related sequencing-based technologies have led to unprecedented progress in the study of microbial evolution in the lab [16], and increasingly have been extended to studying natural environments. Here we will first discuss the purely *passive*, observational role that sequencing has played in earlier investigations following changes in experimental populations (Figure 1A). We follow this with a discussion of how sequencing can provide the key output data for experimental designs where the researcher plays an *active* role in generating variation prior to the initiation of adaptation (Figure 1B).

Sequencing individual isolates reveal evolved genotypes

The most straightforward use of genome sequencing to understand evolution is to determine the complete genome sequence of individual evolved isolates. Researchers using viruses as model systems had been using standard Sanger sequencing for this purpose much earlier [17,18], but the use of 454 sequencing to determine the genetic basis of adaptation in an experiment with *Myxococcus xanthus* [15] was the first in a wave of papers using whole genome sequencing to uncover the genetic bases of adaptation in numerous bacterial systems. This approach provides the number, type, and targets of mutations, and it unambiguously reveals that these mutations are linked together as a genotype (Box 1). Assuming genetic manipulation is possible for the organism of interest, it is then possible to parse apart which of these mutations contribute to these phenotypes. These allelic exchange experiments reveal both specific answers about adaptation of a particular organism to a particular environment, and illuminate general trends about adaptation, such as that beneficial mutations are generally less and less beneficial when present upon backgrounds with higher fitness (i.e., diminishing returns epistasis [19,20]).

Whereas obtaining a single whole genome sequence for an evolved isolate was astonishing in 2006, this has

become absolutely trivial at this point, and the low hurdle for sequencing has tremendously altered the types of scientific questions that can be asked. One great advantage has been the ability to sequence isolates from a tremendous number of independent evolution experiments, thereby obtaining a reasonably-sized sample of what is possible for that strain placed in the selective conditions used. For example, by sequencing isolates from 120 separate populations of *Escherichia coli* evolved to grow at an elevated temperature, it became possible to use the occurrence (or nonoccurrence) of mutations together in the same genotype more (or less) frequently than random expectation to reveal positive (or negative) epistasis between them [21] (Figure 2A). This readily revealed multiple distinct evolutionary trajectories that were possible. If the power of sequencing many isolates is instead directed at multiple isolates from multiple timepoints in a single population, it becomes possible to loosely infer clonal dynamics of these populations [22]. Although it was once thought that beneficial alleles arise and escape drift rarely enough that they would rise in frequency and fix one at a time (i.e., periodic selection, [23]), genomic analyses of isolates (and populations, see below) have made it abundantly clear allele dynamics in populations are tremendously messy due to multiple lineages with beneficial mutations arising at the same time and competing with each other (i.e., clonal interference, [24]). Whereas many of these isolate sequencing studies represented the capstone analysis of already well-studied experimental systems, isolate sequencing has become sufficiently trivial that it can even be used as the first 'gateway' step to decide whether an evolution experiment is worth further experimental inquiry. Indeed, in our own group, multiple times we have only proceeded to pursue a story in greater depth after sequencing results revealed that there was an interesting, unexpected physiological basis to adaptation [25], or a surprising similarity between beneficial mutations arising in different environmental or genetic contexts [26]. Looking forward, assuming the relatively high error rates and large regions lacking coverage common to single-cell sequencing [27,28] can be improved, applying singe-cell approaches to evolved populations would constitute a more extreme version of isolate-based sequencing, in that it would still provide linkage information, but also simultaneously give information about the breadth of genetic diversity in the population.

Metagenomic sequencing of populations uncovers genetic diversity and its dynamics

Just as metagenomics has been applied to directly determine the genomic composition of mixed natural communities, it has become increasingly common to simply sequence the total genomic DNA of evolving populations to sample their diversity across time points and/or replicates. What began with analysis of a single population to determine what fraction of evolved diversity fixed or was lost [29] has matured greatly with increased sequencing depth and new analysis pipelines applied across an evolution experiment [e.g. 30]. The tremendous advantage of metagenomics is that it

samples all alleles present with sensitivity that depends upon depth of coverage and allele abundance, such that alleles that emerge to multiple percent of the population can be confidently identified as not simply being sequence errors. The great challenge, however, is that these data lack linkage information between the detected variants, and thus it is not directly clear which alleles are present on the same genetic background. Instead, additional indirect information – such as the correlation between time points in an evolution experiment – are required for the trajectories of alleles and linkage information to be inferred.

Metagenomic analyses of evolving populations have revealed many insights into the nature of evolutionary dynamics in microbes. For example, work by Lang *et al*. [31] demonstrated that selective sweeps often involved whole cohorts of mutations that had accumulated in a lineage, rather than a series of individual beneficial mutations that arose victorious from clonal interference. A recent paper on the Lenski LTEE populations highlighted lessons metagenomics can reveal over the tremendous timescale of 60,000 experimental generations [30] (Figure 2B). Using extremely fine-scaled temporal coverage of the dozen *E. coli* populations evolved in glucose medium allowed for the direct calculation of quantities such as total mutations along lineages, survival probabilities and transit times of fixed alleles, etc. Furthermore, in this experiment, as was examined over a shorter timescale in an earlier experiment with two growth substrates [32], there were abundant clues in the allele dynamics that it was possible to "sequence ecology" [33]. Despite the overlapping complexities of clonal interference there was evidence of adaptive diversification into two ecotypes [34] occupying separate niches due to the fact that selective sweeps were confined to separate subpopulations within the whole population, indicating non-transitivity and likely negative frequency-dependent fitness interactions.

Lineage tracking reveals fate of many subpopulations simultaneously

In order to uncover the distribution of fitness effects (DFE) possible for a given strain in an environment, it requires accurately quantifying a very large number of (initially) rare lineages across many time points, most of which never rise to more than tiny fractions of a percent of the population, even if it means sacrificing the ability to simultaneously identify the causal mutations that arose to generate those dynamics. In this case, rather than whole-genome sequencing, amplicon sequencing of neutral, barcoded loci focuses the available sequencing depth upon just those tagged sites. Sequencing only a 10^2-10^3 bp stretch containing a barcode signatures, implemented as shorthand for a 10^6-10^7 bp genome, increases the sensitivity of detection by 10^4-10^5 fold [35]. One recent influential paper by Levy *et al*. [36] utilized ~500,000 barcoded lineages to capture the DFE of 25,000 beneficial mutations that occurred during the initial adaptation of yeast to

rich media (Figure 2C). The standard expectation had been that the upper tail of the DFE of beneficial mutations would fall exponentially and monotonically, such that big benefit mutations are uniformly rarer than moderately beneficial ones [37]. Instead, their data suggested that the DFE for beneficial mutation was neither monotonic nor exponential, with the mutations rising to high frequency coming from a small number of discrete peaks in the fitness distribution that occur at substantially higher rates than the exponential expectation. The results of the study also suggested that early adaptive dynamics for the populations investigated were deterministic due to the huge crowd of modestly beneficial mutations that almost never gave rise to lineages that would ultimately be successful but regardless drive increases in mean population fitness, and only later on did stochastic effects become more important (drift, timing of large benefit mutations, occurrence of double mutant combinations). The barcode identifiers can also aid in pulling out the individual winning genotypes, from which standard genome sequencing can reveal the putative causative mutations [38]. Because of its utility for tracking many different variants in tandem, the use of lineage tracking with amplicon sequencing will certainly expand in the future, providing researchers unprecedented speed and depth for probing questions about population dynamics.

Sequencing the fate of variation introduced at sites under selection readily reveals genotype to phenotype mapping

Despite the many advantages to experimental evolution, there is generally no way to control several key features that may have inspired one to be interested in evolving the system that they study in the first place, such as which loci will contain the beneficial mutations that emerge, what types of variation will be exposed to selection, or the simultaneous ability to assess fitness consequences of beneficial, neutral, and deleterious alleles. To explore selection upon a target set of genetic variants, there now exist methods to introduce desired alleles and track their fate simultaneously via amplicon sequencing in a manner analogous to the neutral barcodes described above. At the level of individual genes, this combination of gene synthesis techniques and amplicon sequencing is known as “deep mutational scanning” [39]. This allows the fitness consequence of mutations or mutational combinations to be assessed in parallel via representation in sequencing reads before and after selection, and has been applied across all individual variants of entire proteins, or large subsets of possible mutational combinations [39,40]. Most such experiments run a limited number of generations to assay the fitness consequence of the initial variation that was introduced, but could be allowed to run longer to probe the differential ability to further adapt through additional mutations.

To expand analysis of selection to combinations of alleles at multiple genomic locations, *in vivo* gene editing techniques such as CRISPR/Cas, MAGE, MuGENT, and others can be used to change desired loci across bacterial or

archaeal chromosomes [41-46]. These techniques all allow the researcher to choose where in the genome changes are made, as well as what type of variation is introduced, which is a huge step forward from mutant generation techniques like error-prone PCR that produce random mutational changes. These editing technologies have allowed researchers to alter multiple genes in a single process and assess their effects (Figure 2D), or even to make large scale changes across the genome to unrelated genes and analyze their combined effects [47]. If an efficient screen is available for a phenotype other than fitness, such as the enhancing the production of an industrially valuable compound, such as lycopene [41] or PHB [44], then these techniques can have tremendous biotechnological potential. A current limitation in these techniques, however, is the lack of simple methods to obtain linkage information between the edited loci, although there are some promising approaches being developed [48]. If facile approaches to obtain linkage information from these studies emerge, there will be a tremendous potential to use this approach to map from genotypic to phenotypic landscapes [49], perhaps ultimately leading to greater predictability of evolutionary outcomes related to a given trait [50,51].

Sequencing has allowed evolution in natural populations to be tracked in the same way as laboratory experiments.

Historically, studying microbial evolution outside a laboratory setting has been much less tractable than within, and many of the experimental questions posed could not be effectively executed in natural environments. Sequencing technologies have helped to put studies of natural systems on essentially equal footing with those looking at experimental lab populations. Conversely, this also allows researchers in the lab to construct experiments that are more complex, thus beginning to resemble natural environments or communities [52,53]. One clear way in which this can be seen is in examples from the literature in which time series samples from infections have been taken from patients, where strikingly similar patterns to laboratory experiments have been found for phenomena such as parallelism, rates of molecular evolution, within population dynamics such as clonal interference, etc. [e.g 54-59]. This has frequently been done by sequencing multiple isolates in parallel, but can also be extended to sequencing whole populations obtained from patient samples. In terms of active approaches, barcoded and/or pooled variants can be generated and introduced into infection systems such as animal models to test which genotypes are favored in a host [60,61]. In this way, the inclusion of sequencing has the potential to improve our understanding of disease dynamics, as well as aid in diagnostic evaluations of infections in real time and possibly inform better therapeutic intervention strategies.

It was apparent to those of us who worked upon microbial evolution in the ‘pre-next-generation sequencing’ era that cheaper sequencing was coming, and it would be quite useful, but it would have been hard to envision just how

169 transformative it has been. Now the onus is upon us to design and interpret experiments that maximally utilize the wealth of
170 genomic data are available. Genotypes had been 'losing the battle' to phenotypes in terms of what could be learned, but
171 now genotype-based studies have a seemingly insurmountable lead. Let us hope for similarly revolutionary developments
172 in the ability to assay relevant phenotypes quantitatively and in a high-throughput manner so that it can at last become a fair
173 fight.

174

175 **Figure 1.** Different means for applying sequencing approaches to evolution experiments. A) Passive approaches include
176 isolate as well as metagenomic sequencing to capture information on the diversity of mutations that evolve in experimental
177 populations. Figure adapted from [22]. B) Active approaches arise from methods that allow the generation and/or
178 construction of large numbers of initial variants – neutral barcodes or at loci under selection – and tracking them over time.
179 A short experimental timeframe permits observation of the various rates at which deleterious mutations are lost and neutral
180 mutations will remain at steady frequencies, whereas a longer timeframe will see the neutral mutations begin to be squeezed
181 out by the rising mean fitness of the population, but the relative differences in the beneficial mutational effects become
182 more prominent.

183

184 **Figure 2**. Examples of how sequencing has advanced microbial evolution studies. Panel A: Sequencing of many isolates in
185 tandem allowed tracking of the co-occurrence of mutations, which suggested where genes interact epistatically on fitness
186 (reprinted from [21]). Panel B: Extensive metagenomic sequencing from the Lenski LTEE lineages through 60,000
187 generations of experimental evolution allowed the identification of different sublineages and quantification of selective
188 coefficients for evolved mutations (reprinted from [30]). The presence of co-occurring ecotypes (in bold) was evidenced by
189 a deep divergence between two lineages and selective sweeps only occurring within each of these lineages. Panel C: High-
190 throughput lineage tracking with barcode sequences allowed the quantification of fitness effects for many mutations in
191 parallel, giving a clearer picture of the general distribution of these effects (reprinted from [36]). Panel D: MAGE was
192 implemented to introduce multiplexed genotypic changes into *E. coli* promoters (bottom row) that demonstrated differential
193 effects upon both Indigo production ("phentoype" - middle row) and effects upon both growth ("fitness" - top row)
194 (reprinted from [42]).

195

196 **Box 1. What to expect when you sequence evolved isolates?**

Investigators new to using sequencing as part of their experimental studies are often (justifiably) curious about what they should expect to see from their experimental results. Years of isolate sequencing have provided ample information on a number of general trends that consistently crop up in evolution experiments (many of these were highlighted in [29]), including:

- Observed biases toward non-synonymous changes selected more commonly over synonymous changes within genes.

- More mutations in promoters than expected by chance.

- A high proportion of mutations caused by insertion sequence (IS) element transposition and/or homologous recombination between multiple copies of the same IS.

- Parallelism in the loci containing beneficial mutations between replicate lineages, but generally not to the same site/SNPs. This is especially true for loss-of-function alleles that are beneficial for fitness.

- Patterns of mutations and direct allelic exchange experiments indicate an overwhelming pattern of positive selection upon beneficial mutations, with the exception of strains that become mutators. Mutators display a much wider spectrum of mutational effects observed.

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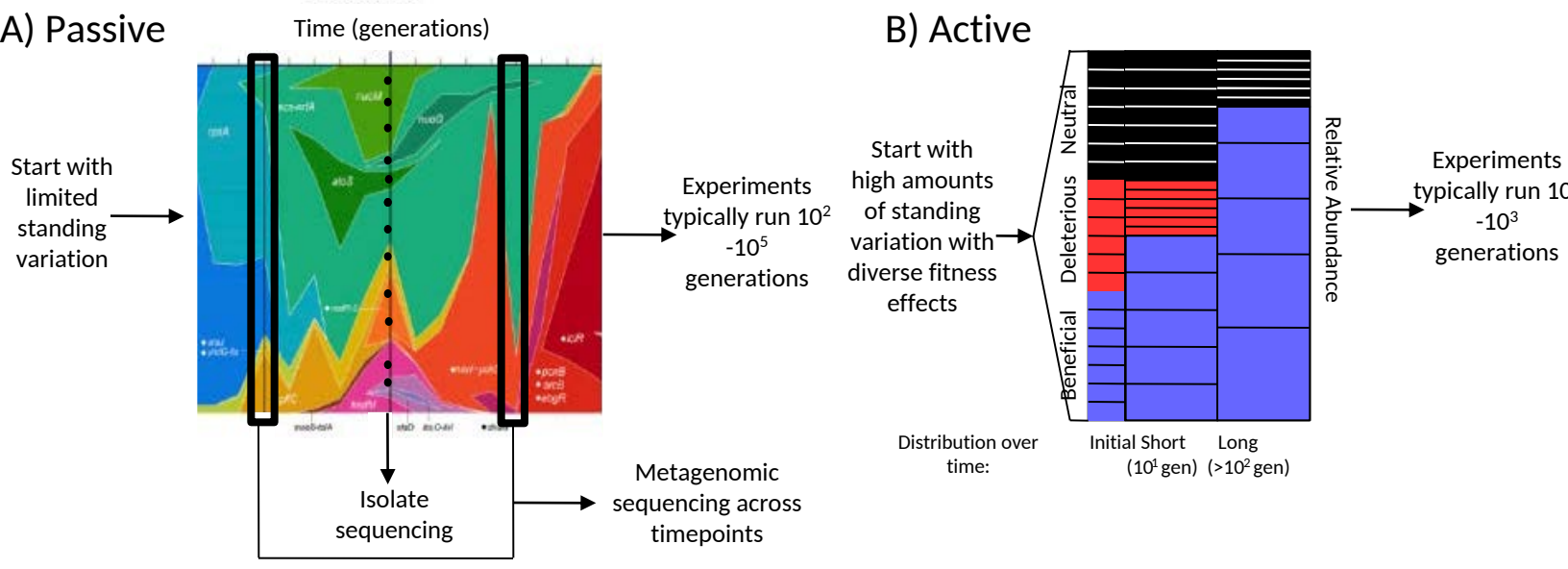
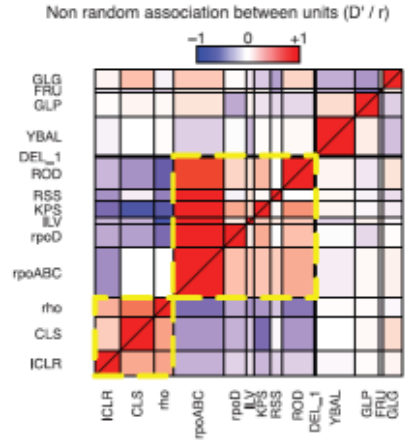
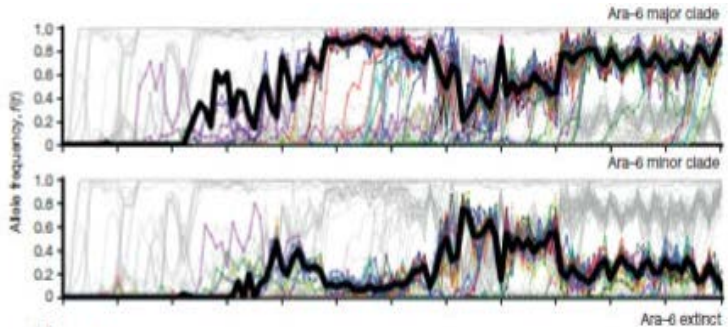

A) Passive
Time (generations)
Start with limited standing variation
Experiments typically run 10^2 -10^5 generations
Isolate sequencing
Metagenomic sequencing across timepoints
B) Active
Start with high amounts of standing variation with diverse fitness effects
Neutral
Deleterious
Beneficial
Relative Abundance
Experiments typically run 10 -10^3 generations
Distribution over time:
Initial Short (10^1 gen)
Long (>10^2 gen)

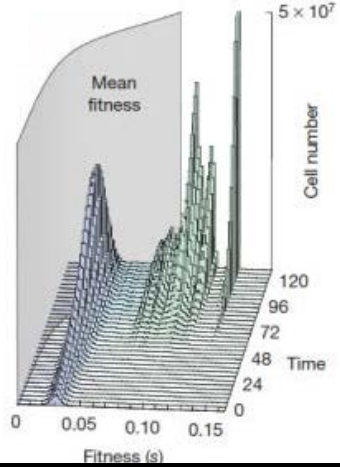
A) Isolate Sequencing



B) Metagenomic Sequencing



C) Lineage Tracking



D) Generation of Variation