

A Population-Based Experimental Model for Protein Evolution: Effects of Mutation Rate and Selection Stringency on Evolutionary Outcomes

Aaron M. Leconte,^{†,‡} Bryan C. Dickinson,[†] David D. Yang,[†] Irene A. Chen,[‡] Benjamin Allen,^{§,||} and David R. Liu^{*,†}

[†]Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138, United States

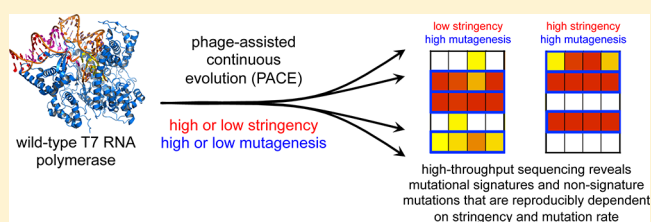
[‡]Department of Chemistry and Biochemistry, University of California, Santa Barbara, California 93106, United States

[§]Program for Evolutionary Dynamics, Harvard University, Cambridge, Massachusetts 02138, United States

^{||}Department of Mathematics, Emmanuel College, Boston, Massachusetts 02115, United States

Supporting Information

ABSTRACT: Protein evolution is a critical component of organismal evolution and a valuable method for the generation of useful molecules in the laboratory. Few studies, however, have experimentally characterized how fundamental parameters influence protein evolution outcomes over long evolutionary trajectories or multiple replicates. In this work, we applied phage-assisted continuous evolution (PACE) as an experimental platform to study evolving protein populations over hundreds of rounds of evolution. We varied evolutionary conditions as T7 RNA polymerase evolved to recognize the T3 promoter DNA sequence and characterized how specific combinations of both mutation rate and selection stringency reproducibly result in different evolutionary outcomes. We observed significant and dramatic increases in the activity of the evolved RNA polymerase variants on the desired target promoter after selection for 96 h, confirming positive selection occurred under all conditions. We used high-throughput sequencing to quantitatively define convergent genetic solutions, including mutational “signatures” and nonsignature mutations that map to specific regions of protein sequence. These findings illuminate key determinants of evolutionary outcomes, inform the design of future protein evolution experiments, and demonstrate the value of PACE as a method for studying protein evolution.



While evolution plays an essential role both in shaping the natural world and in the development of valuable therapeutics, materials, and research tools,^{1–6} the determinants of evolutionary outcomes over long time courses both in nature and in the laboratory remain largely unexplored by systematic experimentation. Experimental efforts to understand protein evolution have largely relied on the reconstruction of presumed evolutionary intermediates^{7–10} or on experimental evolution over modest numbers of rounds of evolution (typically fewer than 10).^{11–15} The time-consuming nature of traditional directed evolution methods has made challenging the study of large, freely evolving, protein populations over long time courses.

In contrast, long evolutionary trajectory experiments have been successfully executed for populations of whole organisms and RNA. Seminal work by Lenski and others studying the evolution of whole organisms through continuous culture^{16–20} has elucidated some of the determinants of organismal evolutionary outcomes, including the effects of population size, the role of epistasis, and the importance of evolvability.^{21–24} Additionally, bacteriophages have been used as a relatively minimal, rapidly reproducing system for experimental evolution at the whole-genome level.^{25,26} Organismal evolution

can be difficult to dissect at a molecular level, however, as mutations typically occur not only in genes of interest but also throughout the host genome. Fitness gains in vivo are therefore frequently influenced by complex sets of mutations, confounding the elucidation of the molecular determinants of fitness gains²⁷ at the protein level. Phage display and related techniques can constrain evolution to a small set of genes of interest, but these methods, being more akin to screening, are generally too cumbersome to support many (e.g., dozens or hundreds of) generations of evolution.²⁸

RNA continuous evolution methods have allowed long evolutionary trajectory experiments on both RNA genomes^{24,25} and catalytic RNAs.^{29–32} These elegant experiments demonstrate the power and potential of continuous evolution methods applied over long time courses. In both cases, the development of the methodology and infrastructure allowing for continuous evolution allowed the study of long evolutionary trajectories.

Received: December 2, 2012

Revised: January 21, 2013

Published: January 29, 2013

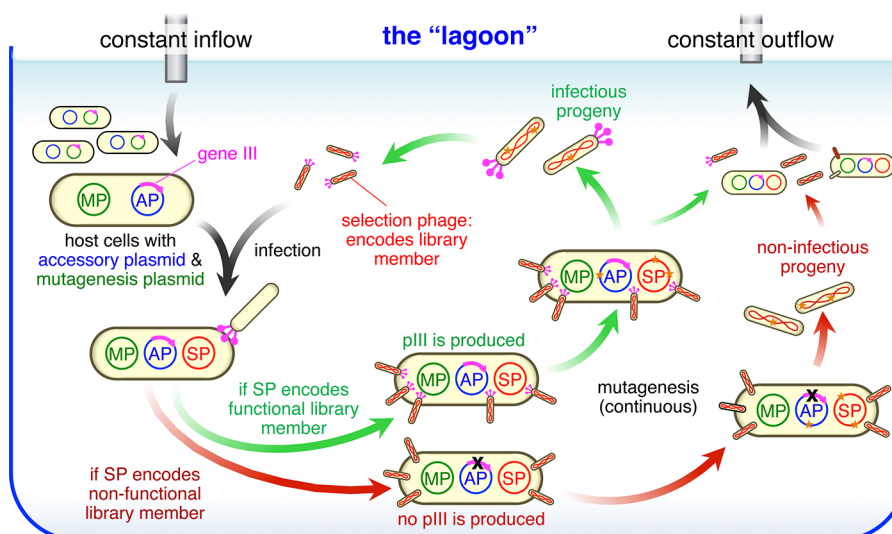


Figure 1. Schematic overview of phage-assisted continuous evolution (PACE). During PACE, selection phage (SP) encoding genes to be evolved propagate in a fixed-volume vessel (a lagoon). The activity of corresponding gene products is linked to the production of an essential phage protein, pIII, encoded by gene III. *E. coli* cells, which contain an accessory plasmid (AP) that is the only source of gene III in the system, are continuously pumped into the lagoon. Only phage genomes encoding active proteins of interest induce gene III expression and trigger the production of viable progeny phage. Because the system is constantly diluted, the ability of phage to persist in the system depends directly on their ability to propagate, which in turn depends on the desired activity of the gene of interest.

However, these methodologies rely on fundamental features of RNA replication and have not been applied to proteins.

Long evolutionary trajectories have not been studied on the single-protein level in part because of a lack of a methodology capable of supporting protein continuous evolution. Recently, we developed phage-assisted continuous evolution (PACE), a method for the continuous directed evolution of proteins³³ that performs the selection, replication, and mutation of genes of interest continuously without human intervention. PACE allows up to ~40 theoretical rounds of evolution to take place every 24 h.³³ The PACE system selectively propagates selection phage (SP) that encode evolving proteins in a continuously diluted fixed-volume vessel (a “lagoon”) by linking the activity of SP-encoded proteins to the production of an essential phage protein, pIII, encoded by gene III. The *Escherichia coli* cells contain an accessory plasmid (AP) that is the only source of gene III in the system (Figure 1). Phage possessing active SP-encoded proteins are capable of generating infectious progeny, while phage possessing inactive SP-encoded proteins are not. Importantly, because of the rate of the continuous dilution, the host *E. coli* cells do not have sufficient time to divide before they exit the lagoon, preventing their evolution and ensuring that only the phage-encoded genes evolve.

The nature of PACE allows mutations to accumulate exclusively in the phage genome. Previous *in vivo* evolution studies have used mutator *E. coli* strains,^{34,35} which introduce mutations throughout both the gene of interest and the *E. coli* genome, complicating the interpretation of fitness gains and necessitating human intervention between rounds. In PACE, the host *E. coli* cells possess an arabinose-inducible mutagenesis plasmid (MP) that is induced only in the lagoon. Like traditional mutator strains, mutations are distributed across the gene of interest and the host. However, unlike traditional mutator strains, mutations persist in the phage genome and not in the *E. coli* host because the average residence time of the *E. coli* cells in the lagoon is insufficient to allow cell division.

The uncoupling of gene-of-interest evolution from host genome evolution during PACE allows the study of large gene populations over hundreds of rounds of evolution in parallel replicates with minimal human intervention. Moreover, the selection conditions of the gene of interest can be carefully controlled with minimal concern for the impact on cell survival or cell evolution. PACE can therefore serve as an experimental platform for studying the determinants of protein evolution outcomes over long evolutionary trajectories.

In this work, we integrated phage-assisted continuous evolution (PACE)³³ and high-throughput DNA sequencing to study the effects of mutation rate and selection stringency on evolving protein populations over long evolutionary trajectories that would be difficult or impractical to implement using conventional directed evolution methods. We observed that specific combinations of mutation rate and selection stringency reproducibly resulted in differences in evolutionary outcomes, including mutational “signatures” and nonsignature mutations that map to specific regions of protein sequence. Our findings illuminate key determinants of protein evolutionary outcomes and suggest hypotheses that inform both the design of future protein evolution experiments and the interpretation of natural protein evolution.

MATERIALS AND METHODS

General Methods. All polymerase chain reactions were performed with Hot Start Phusion II polymerase (Thermo Scientific). Water was purified using a Milli-Q water purification system (Millipore, Billerica, MA). All vectors were constructed by isothermal assembly cloning³⁶ (i.e., Gibson assembly). Single-point mutants and reversions were generated using the QuikChange II site-directed mutagenesis kit (Agilent). All DNA cloning was performed with NEB Turbo cells (New England Biolabs). Plaque assays and PACE experiments were performed using *E. coli* S109 cells derived from DH10B as previously described.³³ Luciferase assays were performed in

NEB 10- β cells (New England Biolabs) as described in the Supporting Information.

Phage Preoptimization. To minimize the potential fitness advantages of mutations to the phage genome, a previously described VCM13 helper phage with T7 RNAP (HP-T7RNAP A)³³ was preoptimized by PACE. HP-T7RNAP A was continuously propagated for 6 days with arabinose induction at a 2.0 volume/h dilution rate using a high-copy number AP-containing gene III under control of a T7 promoter. Wild-type T7 RNA polymerase (T7 RNAP) was then subcloned into a randomly chosen phage backbone clone from this preoptimization selection and sequenced to ensure the correct cloning of the T7 RNAP gene. The resulting SP (SP T7 RNAP wt) was used as the starting point for all PACE experiments.

Phage-Assisted Continuous Evolution (PACE). The turbidostat, lagoons, media, and general PACE setup were set up as previously described.³³ Lagoons had volumes of 40 mL, and the flow rate was 2.0 volumes/h. Lagoon samples were collected at 6, 12, 24, 30, 36, 48, 54, 60, 72, 78, 84, and 96 h. Each lagoon was inoculated with 5×10^4 pfu of SP T7 RNAP wt (see Phage Preoptimization) and propagated continuously for 48 h on AP-T7/T3. To begin the second 48 h of selection (on AP-T3), 40 μ L of lagoon sample from 48 h was used to reinitiate PACE. Each lagoon contained $\sim 10^9$ – 10^{10} phage after 48 h, corresponding to reinitiation with a population size of $\sim 10^6$ – 10^7 phage per lagoon. This large phage population was used to minimize imposing a bottleneck in the evolution between the hybrid promoter and the final T3 promoter while still allowing further experiments with the phage.

Samples used for reselection (the sample from the lagoon that washed out and the samples used for the low-then-high stringency selection) that were more than 1 month old were revived using the following procedure: 40 μ L of lagoon isolate was added to 500 μ L of fresh cells ($OD_{600} = 0.4$), incubated at 37 °C for 30 min, and then added directly to a lagoon to initiate PACE.

High-Throughput Sequencing Data Analysis. A custom MATLAB script (available upon request) was used to align HTS sequencing reads with the wild-type sequence and count the nucleotide and amino acid positions from which the experimental sample deviates from the wild-type sequence. We observed an error rate that varies as a function of nucleotide position; importantly, the error rate is highly reproducible from multiple sequencing runs and sample preparations. We sequenced multiple, independently prepared samples (over multiple sequencer runs) of the wild-type gene and used the error rate of these samples as a “baseline” for future experiments. This yielded both an average error rate and a standard deviation for the error of wild-type sequencing for each nucleotide and amino acid position in the gene (Figure S1 of the Supporting Information).

To compensate for systemic sample preparation and sequencing errors, the observed fraction of mutations at each nucleotide or amino acid position of the wild-type T7-RNAP reference gene was subtracted from the fraction of mutations in a given experimental sample, resulting in the “corrected fraction mutated”. Mutations were defined as amino acid positions with a corrected fraction mutated that is both $\geq 2.5\%$ and at least five standard deviations higher than the corrected fraction mutation of the wild-type reference sequence. Extensive controls demonstrating the validity of this sequencing methodology are detailed in the Supporting Information (see Supplementary Results, Figures S1–S4, and Table S1).

Additional methods are provided in the Supporting Information.

RESULTS

Experimental Design. T7 RNA polymerase (T7 RNAP) is a single-subunit RNA polymerase that recognizes the native T7 promoter with a high degree of specificity.³⁷ We used PACE to evolve T7 RNAP to recognize the T3 promoter^{38,39} (Figure 2A), which is not natively recognized by T7 RNAP, under four

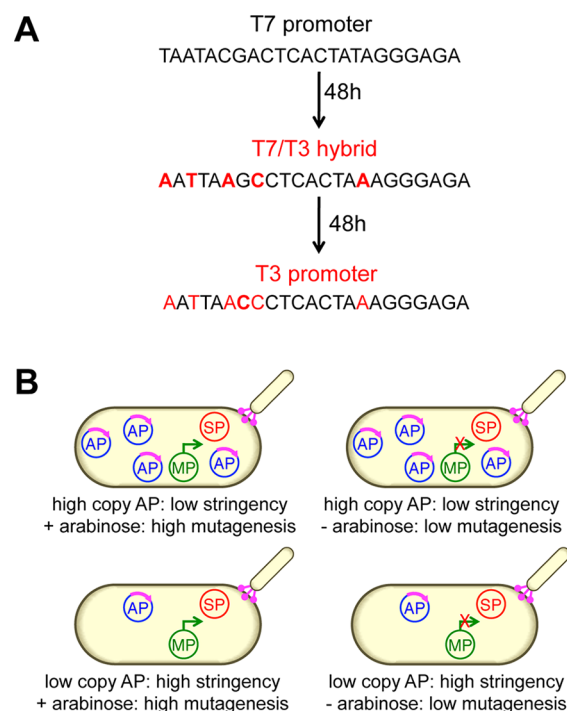


Figure 2. T7 RNAP promoter evolution as a model for studying the effects of mutation rate and selection stringency on protein evolution. (A) DNA sequence of the T7 promoter, the T7/T3 hybrid promoter, and the final T3 promoter target of the evolution. (B) Schematic of the experimental parameters varied in this study. Stringency was varied by controlling the copy number of the accessory plasmid (AP), and mutagenesis was varied by inducing the expression of mutagenic genes on the mutagenesis plasmid (MP).

distinct selection conditions, each in 4-fold replicate: high stringency and high mutagenesis, high stringency and low mutagenesis, low stringency and high mutagenesis, and low stringency and low mutagenesis. We controlled selection stringency by modulating the copy number of the AP, which modulates the concentration of substrate DNA within the cell. We previously demonstrated that infectivity of phage progeny, and thus selection stringency, can be modulated by changing the copy number of the AP.³³ The high-copy number AP (pMB1 Δ rop origin) is present in approximately 20–60 copies per cell⁴⁰ and corresponds to “low-stringency” selection conditions, while the low-copy number AP (SC101 origin) is present at a level of approximately five copies per cell⁴¹ and corresponds to “high-stringency” conditions. The mutagenesis rate was modulated using an inducible mutagenesis plasmid (MP), which enhances the mutation rate of propagating SP by ~ 100 -fold (Figure 2B).³³ All high-mutagenesis PACE lagoons contained 1% arabinose, which increases the mutation rate of the phage produced by approximately 100-fold, sufficient to generate all possible double mutants of T7 RNAP shortly after

the gene enters the lagoon. The low-mutagenesis lagoons received an equivalent volume of water and therefore relied on the basal mutation rate of DNA replication ($\sim 5 \times 10^{-7}$ per nucleotide per generation⁴²) to generate diversity.

All selections began by seeding each lagoon with 5×10^4 pfu of SP encoding wild-type (wt) T7 RNAP. Because wild-type phage do not propagate on host cells containing the T3 promoter, the lagoons were continuously evolved for 48 h on a hybrid T7/T3 promoter (AP hybrid)³³ that served as an evolutionary steppingstone to T3 promoter recognition. A sample from each lagoon was then diluted into a fresh lagoon receiving host cells harboring AP-T3 and continuously evolved for an additional 48 h (96 h total). Phage surviving 96 h of PACE in each lagoon will have undergone an average of ~ 100 theoretical rounds of evolution,³³ calculated on the basis of the theoretical time for an average phage life cycle during PACE, and survived an $\sim 10^{84}$ -fold net dilution.

Genetic Evidence of Positive Selection. To quantitatively analyze population genotypes, we subjected lagoon samples to high-throughput DNA sequencing (HTS). We experimentally demonstrated that HTS could reliably detect mutations present at a $\geq 2.5\%$ frequency in each population (Supplementary Results, Figures S1–S4, and Tables S1 and S5 of the Supporting Information). Across all lagoons, 153 instances of significantly mutated nucleotide positions were observed; of these, 101 represent coding mutations, while 52 represent silent mutations. Of the 101 coding mutations, 32 are observed in more than one lagoon (32%) while only one of the noncoding mutations is observed in multiple lagoons (2%).

The 101 coding mutations result in mutations at 97 of the 883 amino acids of T7 RNAP, representing 11% of the total amino acids of the protein. Among these are a number of mutations that have been previously described to be important for substrate broadening, such as E222K,³⁸ to serve as a specificity determinant for T3 promoter recognition such as N748D,³⁹ or have been identified in previous work, such as G542V.⁴³ Collectively, the strong enrichment of coding mutations over noncoding mutations, the recurrent nature of these mutations, and the observation of known beneficial mutations provide compelling evidence of positive evolution.

All Four Selection Conditions Evolve T3 Promoter Recognition Activity. SPs encoding wt T7 RNAP do not form plaques on host cells containing either low- or high-stringency AP-T3. In contrast, 15 of the 16 lagoons at 96 h contained phage that formed plaques on AP-T3 of their respective stringency. Although all 16 lagoons yielded phage that were active on the T7/T3 hybrid promoter at the conclusion of the T7/T3 hybrid selection (48 h time point), one lagoon repeatedly failed to yield T3-active phage (high-stringency, low-mutagenesis lagoon 1) at the end of the T3 selection, likely because of its distinct genetic composition following T7/T3 hybrid evolution (see below).

We assayed the activity of 10 or more RNAP genes from each of the 15 active 96 h lagoons. Although wt T7 RNAP showed no detectable activity on the T3 promoter (less than $\sim 1\%$), the average lagoon from all 15 active lagoons at 96 h exhibited activities on the T3 promoter of $\geq 11\%$ of the activity of wt T7 RNAP on the T7 promoter, which we define as 100% (Figure 3). Notably, RNAP variants evolved in the high-stringency lagoons showed an average T3 promoter activity of 215%, whereas the low-stringency lagoons evolved an average T3 promoter activity of 43%. These results indicate that

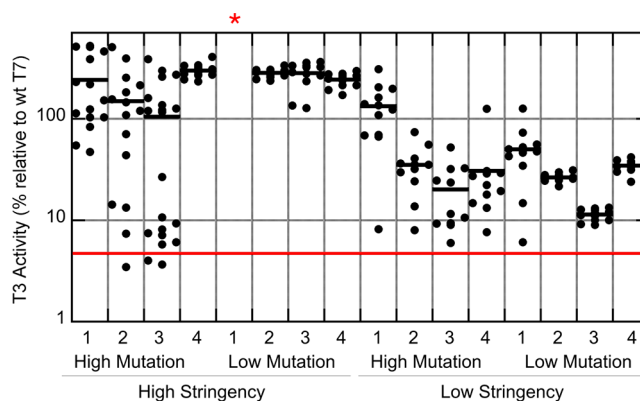


Figure 3. T3 promoter recognition activity of each lagoon after continuous evolution for 96 h. Each dot represents the relative transcriptional activity of a single randomly chosen clone on the T3 promoter in reporter *E. coli* cells. The black bars represent the average activity of all the assayed clones from one lagoon. The red line represents the endogenous background level of expression of the T3 promoter without any exogenous RNAP. High-stringency, low-mutagenesis lagoon 1 resulted in no surviving RNAP genes and is identified with a red asterisk.

evolved activity levels were strongly dependent on selection stringency.

Potential Explanation for Phage Washout of High-Stringency, Low-Mutagenesis Lagoon 1. To test if the inability of high-stringency, low-mutagenesis lagoon 1 to survive the final 48 h of selection on AP-T3 was a stochastic occurrence or instead reflected a property of this lagoon's population after 48 h, we repeated the final 48 h of T3 selection for this lagoon in duplicate. Once again, no active phage were observed in any replicate after 48 h of high-stringency, low-mutagenesis selection on AP-T3, indicating that the enzymes at the end of the 48 h T7/T3 hybrid selection in this lagoon were not capable of evolving sufficient activity on the T3 promoter.

To begin to understand the inability of one of the lagoons to complete the selection at low mutagenesis and high stringency, we performed HTS on samples from all of the lagoons at 48 h using the methods described above. When we compared genetic data from 48 h samples to genetic data from 96 h samples, we noticed that mutation at E222 varied significantly between 48 and 96 h (Figure S5 of the Supporting Information). At 48 h, a wide range of mutations was present at E222, while at 96 h, only E222K and E222Q were observed. Thus, several of the mutations present at 48 h are de-enriched upon selection for activity on T3. One of those de-enriched mutations, E222G, is present in 100% of the population of the lagoon that did not complete the selection. Importantly, while three of the eight high-stringency populations possess E222G as a measurable subpopulation at 48 h, zero possess E222G at 96 h.

We hypothesized that E222G may be unable to achieve high activity in the context of the other highly enriched mutation of the high-stringency, low-mutagenesis conditions, N748D. To test this hypothesis, we biochemically reconstructed the three possible combinations of E222K, -Q, or -G and N748D and assayed their activity. We observed that the E222K/N748D pair (the most common combination) is the most active double mutant on T3, while the E222G/N748D pair was the least active double mutant on T3 (Figure S6 of the Supporting Information). While the E222G/N748D pair is more active

than N748D alone, it is still less active than the other double mutants and may not possess sufficient activity to propagate. This could be an example of negative sign epistasis, in which mutations that are individually beneficial become deleterious in combination, which have been previously identified¹² and may have created evolutionary dead ends. Other possible explanations for the relationship between E222G and phage washout include increased polymerase promiscuity or decreased protein stability, which are beyond the scope of this study. We also note that while our observations of E222G's abundance and effects on activity are suggestive, other, less obvious genetic differences might also underlie the inability of phage in this lagoon to propagate.

Notably, there is significantly more amino acid variation at E222 at 48 h among high-mutagenesis, high-stringency lagoons than among low-mutagenesis, high-stringency lagoons. While several other lagoons contained a measurable subpopulation containing E222G at 48 h, in each case the E222G mutation was not present at 96 h (Figure S5 of the Supporting Information). Unlike the lagoon that did not yield viable phage at 96 h, each of the other lagoons that contained E222G at 48 h also contained a subpopulation containing either E222K or E222Q, which, upon selection on T3, overtook the population. These findings suggest that, particularly under low-mutagenesis conditions, different replicates can lead to dramatically different outcomes, including the inability to complete an evolutionary trajectory.

Selection Conditions Determine the Genotypic Outcome. Populations of RNAP genes obtained through HTS data were characterized using three metrics that reflect different aspects of genetic diversity and one new metric that we developed to reflect evolutionary divergence and reproducibility. M is the total number of unique mutations ($\geq 2.5\%$ frequency in the population) present in the set of four lagoon replicates for a given selection condition; $\langle M \rangle$ is the average number of mutations in each of the four lagoon replicates across a given selection condition, and ISI (inverse Simpson index, averaged across loci) measures the average genetic diversity over all populations.⁴⁴ To measure the similarity of evolved populations, we developed a new metric, F_{div} (Supporting Information), which is based on F_{ST} ⁴⁵ but is modified to reflect the divergence between populations relative to the divergence from an ancestral starting gene. For the purpose of detecting divergent or parallel evolution from a known ancestor, F_{div} has the advantage that, if a mutation at a particular locus fixes in two separate populations, this will decrease the value of F_{div} , indicating greater parallelism, relative to the case in which neither population has a mutation at this locus. In contrast, F_{ST} treats these two cases as equivalent. As with F_{ST} , compared populations are increasingly divergent and dissimilar as F_{div} approaches 1.

The highest values for all three diversity indices (Table 1) were observed for the low-stringency, high-mutagenesis condition, and the lowest values were observed for the high-stringency, low-mutagenesis condition, indicating that higher mutation rates and lower selection stringency increase the mutational diversity of evolved populations, as expected. We calculated F_{div} for each selection condition and for each possible pair of selection conditions to compare the similarity between sibling lagoons (those evolved under identical conditions) with the similarity of nonsibling lagoons (Figure 4 and Table S2 of the Supporting Information). Among the 10 F_{div} values, the three smallest F_{div} values were from populations within a given

Table 1. Values of M (the total number of unique mutations present in the set of four lagoon replicates for a given selection condition), $\langle M \rangle$ (the average number of mutations in each of the four lagoon replicates across a given selection condition), and ISI (the average genetic diversity over all populations measured by inverse Simpson index, averaged across loci) for All Selections^a

| mutagenesis rate | stringency | M | $\langle M \rangle$ | ISI |
|------------------|------------|-----|---------------------|---------------------|
| high | low | 64 | 24 ± 10 | 1.0068 ± 0.0008 |
| low | low | 12 | 4 ± 2 | 1.0029 ± 0.0008 |
| high | high | 41 | 15 ± 5 | 1.0045 ± 0.0008 |
| low | high | 5 | 3 ± 0 | 1.0012 ± 0.001 |

^aThe error in $\langle M \rangle$ is the standard deviation. The error in ISI was calculated according to the description in Materials and Methods.

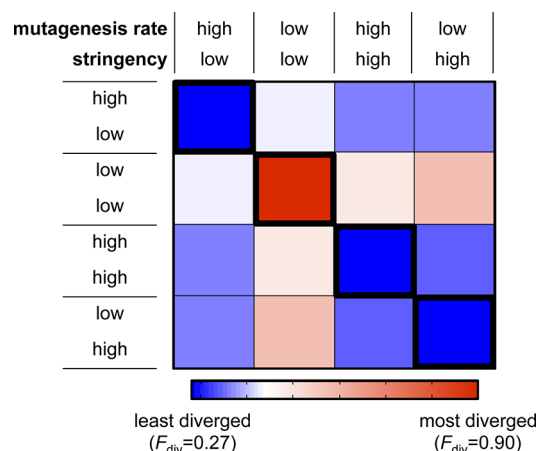


Figure 4. Genotypic outcomes of RNAP evolution under different conditions. F_{div} values at 96 h, calculated for each set of four sibling lagoons and compared to each other set of four nonsibling lagoons.

selection condition (for low stringency and high mutagenesis, $F_{\text{div}} = 0.29$; for high stringency and high mutagenesis, $F_{\text{div}} = 0.27$; for high stringency and low mutagenesis, $F_{\text{div}} = 0.29$). These results indicate that the most similar evolutionary outcomes occurred within one set of selection conditions, implying that selection condition similarity is the primary determinant of the relatedness of evolutionary outcomes. Induction of mutagenesis under low- or high-stringency selection increased all three metrics of diversity compared to those of low-mutagenesis populations but surprisingly resulted in lower F_{div} values under low stringency. This finding indicates that an increased level of mutagenesis results in increased evolutionary convergence of populations relative to the genetic distance separating them from their common ancestor.

Selection Conditions Generate Distinct Mutational Signatures. With the exception of mutations at E222, most high-abundance mutations, defined as those that appear in at least 50% of a lagoon's population, are observed in multiple sibling lagoons but occur only rarely within lagoons subjected to other selection conditions. The similarity between the high-abundance mutations in sibling lagoons creates a common set of convergent mutations, a "mutational signature", unique for that selection condition (Figure 5). For example, the populations evolved under high-stringency, high-mutagenesis conditions contain E222K and N748D at $\sim 100\%$ abundance and Y178H, -C, or -D at 57% abundance. This particular pattern of mutations is absent in lagoons subjected to the other

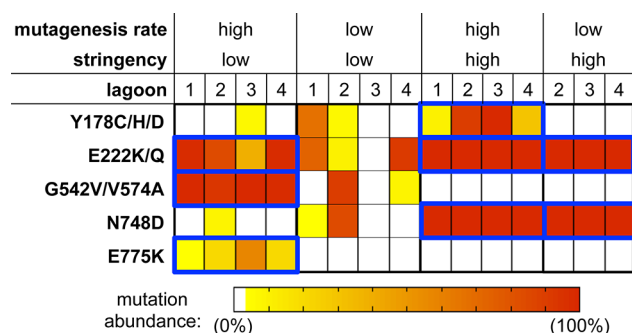


Figure 5. Mutational frequency of positions mutated in at least 50% of any population demonstrating distinct mutational signatures that depend on selection conditions.

three selection conditions. Genes evolved under high-stringency, low-mutagenesis conditions possess a related though distinct signature that lacks a mutation at Y178 (100% mutation of E222K or -Q and N748D). The low-stringency, high-mutagenesis populations evolved a different signature of E222K, E775K, and either G542V or V574A, which appear to be negatively epistatic (Figures S7 and S8 of the Supporting Information). Each of these three mutational signatures is common to sibling lagoons but was not found in any of the 12 nonsibling lagoons. As suggested by its high F_{div} value (0.90, the highest observed value), no signature mutations emerged from the low-stringency, low-mutagenesis selection conditions, even though enzymes surviving these conditions evolved significant T3 promoter activity.

Nonsignature Mutations Converge on Specific Amino Acid Regions. Although the reproducibility of the mutational signatures is striking, the vast majority (91%) of mutations are not found in a mutational signature. These nonsignature mutations (NSMs) are present at <50% abundance, and many (30%) evolved independently in multiple lagoons. While these NSMs did not strongly converge on specific amino acids, we wondered if these mutations converged on particular regions of primary amino acid sequence. To test this possibility, we computationally generated 1000 sets of simulated protein sequences with a total mutational frequency identical to that of the 15 lagoons of 96 h but with these mutations randomly distributed throughout the simulated proteins. We then tested if the experimentally observed NSMs are more frequently

clustered within 10-amino acid segments covering the entire protein than in the simulated sequences (Supporting Information).

We observed that the experimental data set has a greater number of 10-amino acid segments containing five or more NSMs than any of the 1000 simulated sequences ($p < 0.001$), suggesting that the observed mutations are more tightly grouped than would be expected by random chance. There are nine different overlapping protein segments (“clusters”) that contain five or more mutations (Table S3 of the Supporting Information). On the basis of the crystal structure of the initiation phase T7 RNAP,^{46,47} which is thought to be the most important for promoter recognition and clearance, four of these windows (amino acids 121–136, 230–247, 379–394, and 763–779) are predicted to make direct contact with either the DNA substrate, the nascent RNA transcript, or the incoming NTP. Perhaps more surprisingly, five of these amino acid regions (56–76, 153–184, 453–469, 595–607, and 672–697) do not make direct contacts with the substrate in the initiation phase T7 RNAP structure, but all but one of these regions (453–469) makes DNA or RNA contacts in the elongation structure,⁴⁸ suggesting optimization of these regions may improve polymerase parameters aside from promoter clearance.

To analyze whether NSM clusters are specific to selection conditions, we repeated the simulations described above for each selection condition separately (Supporting Information). Although we did not identify clusters that were specific to either of the two low-mutagenesis selections, three clusters are specific to the high-stringency, high-mutagenesis selection conditions and three different clusters are specific to the low-stringency, high-mutagenesis conditions. Interestingly, in five of these six clusters, we observed significantly enriched mutations as a function of selection stringency (Figure 6A,B). These results suggest that NSMs tend to converge on specific regions of primary sequence that are dependent on selection stringency and therefore experimentally demonstrate that selection conditions can cause convergence on mutations in distinct regions of primary sequence.

Stringency Defines Distinct Mutational Paths through the Fitness Landscape. The differences in both the mutational signature and the NSM clusters in the populations selected at high and low stringency suggest that the low-stringency solutions may not be on the same mutational path⁴⁹

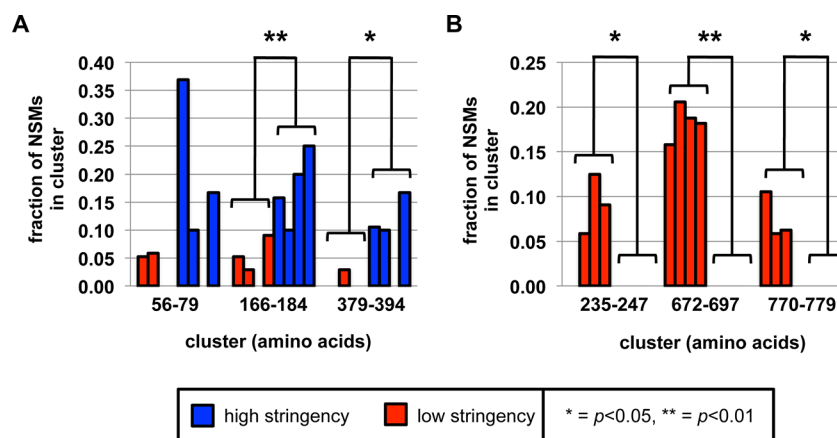


Figure 6. Clustering of nonsignature mutations (NSMs). (A) Fraction of NSMs identified in specific clusters in the high-stringency populations. (B) Fraction of NSMs identified in specific clusters in the low-stringency populations.

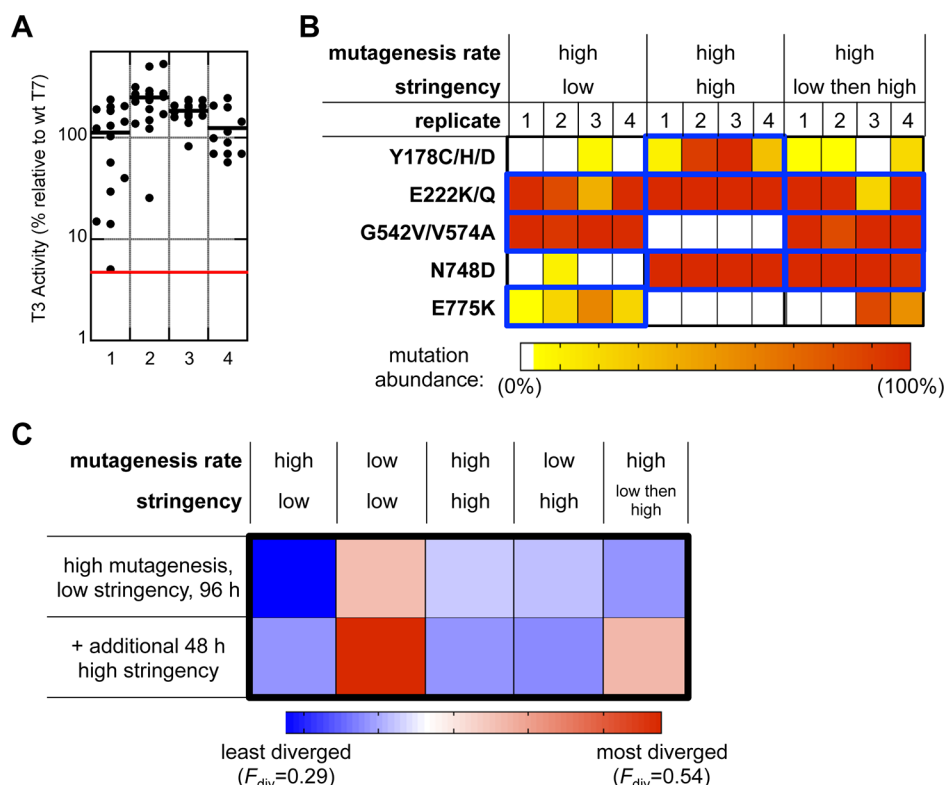


Figure 7. Low-then-high stringency evolution results in distinct genotypes. (A) T3 promoter recognition activity of low-stringency, high-mutation lagoons after continuous evolution for an additional 48 h under high-stringency, high-mutagenesis conditions. Each dot represents the relative transcriptional activity of a single randomly chosen clone on the T3 promoter in reporter *E. coli* cells. The black bars represent the average activity of all the assayed clones from one lagoon. The red line represents the endogenous level of transcription of the T3 promoter in *E. coli* without any expressed RNAP. (B) Abundance of mutations present in at least 50% of any population, demonstrating that mutational signatures are dependent on selection stringency history. (C) F_{div} values calculated for the low-stringency, high-mutation lagoons at 96 h and the low-stringency, high-mutation lagoons after continuous evolution for an additional 48 h under high-stringency, high-mutagenesis conditions compared to all other sets of populations at 96 h.

as their high-stringency counterparts. These results are consistent with a model in which at least two distinct mutational paths through the fitness landscape, one associated with high activity and one associated with lower activity, are accessed in a stringency-dependent manner. To test this hypothesis, lagoon samples from all four of the 96 h low-stringency, high-mutagenesis lagoon replicates were separately evolved on AP-T3 for an additional 48 h under high-stringency, high-mutagenesis conditions (“low-then-high stringency”). If the resulting populations converge on the genotypes of the populations evolved directly under high-stringency, high-mutagenesis conditions, then these results would suggest that both conditions guided the populations through similar mutational fates. Alternatively, if the resulting genotypes remain segregated on the basis of selection stringency history, this result would suggest that the populations followed different evolutionary paths to reach a final high-activity end point.

While only one of the four low-stringency, high-mutagenesis lagoons had average activity on the T3 promoter of >35% at 96 h, all four of the lagoons evolved at least 115% average activity after high-stringency selection for an additional 48 h, very similar to the activity levels of the original 96 h high-stringency populations (Figure 7A). Despite the similar phenotypes of the high-stringency and the low-then-high stringency populations, the resulting populations still possess strong genotypic differences. The low-then-high stringency populations possess a mutational signature consisting of E222K, G542V/V574A,

and N748D, different than the mutational signature of any other selection condition (Figure 7B). M , F_{div} , and ISI of the newly evolved populations increased while $\langle M \rangle$ remained the same, indicating an increase in diversity and divergence without an increase in the number of average mutations per lagoon (Table S4 of the Supporting Information). F_{div} between the new population and both low-stringency selections increased, while F_{div} between the new population and both of the high-stringency selections decreased (Figure 7C and Table S4 of the Supporting Information), indicating that the population became more high-stringency-like during the course of the selection but still retained significant low-stringency character. The observation that the low-then-high stringency selection resulted in a different genotype than the high-stringency selections suggests that the low-stringency mutational signature arises from a distinct mutational path through the fitness landscape.

DISCUSSION

PACE supports multiple parallel lagoons each containing up to $\sim 10^9$ different mutants in a gene of interest, minimizes population bottlenecks, and allows hundreds of theoretical rounds of evolution in multiple replicates to be performed on a practical time scale (days to weeks). Coupled with HTS, PACE allows population-level studies of protein evolution. The tunable nature of our system allowed us to vary several fundamental parameters of evolution (selection stringency and

mutation rate) and examine how each affected phenotypes, genotypes, and the reproducibility of those genotypes. Our findings reveal in molecular detail that changing the mutation rate, selection stringency, or the schedule of selection pressures can result in distinct and reproducible population-wide genetic differences. PACE provides an opportunity to directly observe protein evolution in real time on the population level, which could help validate the knowledge gained from previous theoretical models and experimental systems. We note, however, that because the conditions of any laboratory evolution experiment, including the ones described here, differ from those found in other experiments or in nature, the extent to which these effects will be consistently observed across various protein evolution systems remains to be tested.

Families of related solutions (mutational signatures) reproducibly emerged as a function of selection conditions, and changing either the mutation rate or the selection stringency resulted in a unique mutational signature. Mutations at E222, which were highly enriched under all selection conditions except the low-stringency, low-mutagenesis condition, are known to broaden substrate specificity.³⁸ Both high-stringency conditions enriched N748D, a known specificity determinant for T3 promoter recognition³⁹ that is found in native T3 RNA polymerase and makes direct contacts with the DNA promoter at the T7 RNAP specificity loop.⁴⁶ Accessing high-T3 promoter activity regimes, as was observed with both high-stringency conditions, may require this direct T3 promoter–RNAP interaction. In further support of this hypothesis, all four populations that went through the low-then-high stringency selection conditions enriched N748D as they achieved high activity on the T3 promoter. While the experiments presented here involved a wider bottleneck than many previous studies (to $\sim 10^7$ phage per lagoon), the role of this decrease in population size on the genotypic outcomes is unknown. Similar PACE experiments can be used in the future to study the effects of different-sized bottlenecks on evolutionary outcomes by seeding the T3 promoter evolution with different amounts of phage from the hybrid promoter lagoons.

The high-stringency, high-mutagenesis condition also enriched mutations at Y178, which makes potential DNA contacts in the elongation complex.⁵⁰ This mutation, which was not enriched in the high-stringency, low-mutagenesis condition, may provide additional fitness advantages not directly observed in the transcriptional reporter assay. For example, mutations at Y178 may confer increased stability and therefore increased tolerance of the mutational load experienced by populations under high-mutagenesis conditions.^{51,52}

Low-stringency, high-mutagenesis conditions resulted in the unique enrichment of E775K, which makes contacts with DNA in the initiation complex,⁴⁶ and either G542V, which is located near the DNA substrate and has previously been identified as a mutation associated with broadened ribonucleotide substrate scope,⁴³ or V574A. These combinations of mutations resulted in lower average T3 activity than those populations with N748D. Intriguingly, the low-stringency, high-mutagenesis populations scored the highest on all measures of diversity, including the total number of unique mutations, the average number of mutations, and the inverse Simpson index. Therefore, this unique mutational signature is likely not a result of these populations simply evolving less. Instead, it appears that the combination of G542V/V574A and E775K provides additional benefits under low-stringency, high-mutagenesis conditions, even though that combination has lower

measured T3 promoter activity than those populations with N748D. Consistent with this model, E775K was generally de-enriched when the low-stringency, high-mutagenesis populations were evolved under high-stringency conditions. This observation also suggests a potential epistatic interaction between N748D and E775K, even though these residues are not located in the proximity of one another.

Given that T3 promoter recognition activity strong enough to survive the high-stringency selection should also pass the low-stringency selection, it is not clear why the mutational signature in the low-stringency, high-mutagenesis selections reproducibly differs from that of the high-stringency, high-mutagenesis conditions. The signatures obtained in these two conditions not only differ but also have very few residues in common; only E222K is observed in both mutational signatures. These observations suggest that the low-stringency signature is not merely a steppingstone to the high-stringency signature but instead represents a unique genotype. The high reproducibility of these two mutational signatures argues against stochasticity as the basis for their differences and instead suggests that they may make important contributions to aspects of fitness beyond simply increasing T3 promoter transcription levels.

Induction of mutagenesis resulted in increased diversity, as expected, but also increased the reproducibility of evolution. This effect may arise for a variety of reasons. (1) Many possible solutions may satisfy the selection criteria, in which case enhanced mutational sampling may allow more frequent access to a smaller set of superior solutions. (2) Increasing the mutation rate may implicitly select for the ability to tolerate additional mutations, and satisfying this additional constraint might contribute to the decreased observed divergence. (3) High mutational loads may result in a high fraction of completely inactivated genes, resulting in narrow bottlenecks of surviving genes and sequence convergence.^{53,54} The precise molecular underpinnings and potential biological relevance of this observation merit future investigation.

Increasing the selection stringency also significantly influenced evolutionary outcomes, resulting in fewer mutations and lower diversity, but more reproducible results. These outcomes are consistent with a smaller set of possible outcomes that satisfy the criteria of the selection and a more constrained fitness landscape.

We also observe nonsignature mutations (NSMs) that vary both between and within selection conditions and converge on regions of primary sequence in a selection condition-dependent manner (clusters). It is tempting to speculate that these mutations may be the result of functional redundancy or epistasis with the signature mutations. For example, clustered NSMs might have redundant functions of optimizing positioning or folding of a piece of secondary structure altered by the presence of a beneficial, but not optimal, signature mutation, as has been hypothesized in the “evolutionary Stokes shift” theory.⁵⁵ The enrichment of these stabilizing, compensatory mutations is critical at high mutation rates⁵⁶ and in the acquisition of new functions.⁵⁷ This further optimization is consistent with the prevalence of clusters predicted to make substrate contacts in the elongation phase of T7 RNAP, but not in the initiation phase. Regardless of their origin, this significant and curious phenomenon would be difficult to observe without many parallel replicates as well as HTS, suggesting that further application of PACE as an evolutionary model system might

elucidate important additional insights into molecular evolution.

These findings have implications for future protein evolution experiments. Our observation that different signature and nonsignature mutational solutions evolve in response to changes in mutation rate, selection stringency, and stringency history suggests that performing parallel laboratory evolution experiments under varying mutagenesis levels, stringencies, and stringency schedules may yield a broader diversity of evolved solutions than a more conventional approach of increasing selection stringency of a single population as the number of completed rounds of evolution increases. In addition, because a common goal of some early stage laboratory protein evolution efforts is to generate a diverse set of modestly active variants prior to a later stage in which these variants are recombined and compete,⁵⁸ our results suggest that low-stringency, low-mutagenesis conditions—conditions that resulted in this study in a broad, consensus-free population of modestly active variants—are well-suited to the early stages of evolution, while increasing stringency or increasing mutagenesis can help drive early stage populations to high-activity consensus.

■ ASSOCIATED CONTENT

■ Supporting Information

Supplementary methods, supplementary discussion, Figures S1–S8, and Tables S1–S5. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: (617) 496-1067. E-mail: drliu@fas.harvard.edu.

Present Address

[†]W. M. Keck Science Department of Claremont McKenna, Pitzer, and Scripps Colleges, Claremont, CA 91711.

Author Contributions

A.M.L. and B.C.D. contributed equally to this work.

Funding

This research was supported by DARPA HR0011-11-2-0003, DARPA N66001-12-C-4207, and the Howard Hughes Medical Institute. A.M.L. was supported by National Institutes of Health National Research Service Award Postdoctoral Fellowship F32GM095028. B.C.D. is a Fellow of the Jane Coffin Childs Memorial Fund for Medical Research. B.A. is supported by the Foundational Questions in Evolutionary Biology initiative of the John Templeton Foundation. D.R.L. is an Investigator with the Howard Hughes Medical Institute.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Kevin Esvelt and Jacob Carlson for materials and helpful discussions.

■ ABBREVIATIONS

PACE, phage-assisted continuous evolution; HTS, high-throughput sequencing; SP, selection phage; AP, accessory plasmid; MP, mutagenesis plasmid; RNAP, RNA polymerase; wt, wild-type; *M*, total number of unique mutations; $\langle M \rangle$, average number of mutations; ISI, inverse Simpson index; NSM, nonsignature mutation.

■ REFERENCES

- (1) Vasserot, A. P., Dickinson, C. D., Tang, Y., Huse, W. D., Manchester, K. S., and Watkins, J. D. (2003) Optimization of protein therapeutics by directed evolution. *Drug Discovery Today* 8, 118–126.
- (2) Turner, N. J. (2009) Directed evolution drives the next generation of biocatalysts. *Nat. Chem. Biol.* 5, 567–573.
- (3) Hancock, S. M., Rich, J. R., Caines, M. E., Strynadka, N. C., and Withers, S. G. (2009) Designer enzymes for glycosphingolipid synthesis by directed evolution. *Nat. Chem. Biol.* 5, 508–514.
- (4) Gupta, R. D., Goldsmith, M., Ashani, Y., Simo, Y., Mullokandov, G., Bar, H., Ben-David, M., Leader, H., Margalit, R., Silman, I., Sussman, J. L., and Tawfik, D. S. (2011) Directed evolution of hydrolases for prevention of G-type nerve agent intoxication. *Nat. Chem. Biol.* 7, 120–125.
- (5) Bornscheuer, U. T., Huisman, G. W., Kazlauskas, R. J., Lutz, S., Moore, J. C., and Robins, K. (2012) Engineering the third wave of biocatalysis. *Nature* 485, 185–194.
- (6) Bawazer, L. A., Izumi, M., Kolodin, D., Neilson, J. R., Schwenzer, B., and Morse, D. E. (2012) Evolutionary selection of enzymatically synthesized semiconductors from biomimetic mineralization vesicles. *Proc. Natl. Acad. Sci. U.S.A.* 109, E1705–E1714.
- (7) Lunzer, M., Miller, S. P., Felsheim, R., and Dean, A. M. (2005) The biochemical architecture of an ancient adaptive landscape. *Science* 310, 499–501.
- (8) Weinreich, D. M., Delaney, N. F., Depristo, M. A., and Hartl, D. L. (2006) Darwinian evolution can follow only very few mutational paths to fitter proteins. *Science* 312, 111–114.
- (9) Dean, A. M., and Thornton, J. W. (2007) Mechanistic approaches to the study of evolution: The functional synthesis. *Nat. Rev. Genet.* 8, 675–688.
- (10) O'Maille, P. E., Malone, A., Dellas, N., Andes Hess, B., Jr., Smentek, L., Sheehan, L., Greenhagen, B. T., Chappell, J., Manning, G., and Noel, J. P. (2008) Quantitative exploration of the catalytic landscape separating divergent plant sesquiterpene synthases. *Nat. Chem. Biol.* 4, 617–623.
- (11) Romero, P. A., and Arnold, F. H. (2009) Exploring protein fitness landscapes by directed evolution. *Nat. Rev. Mol. Cell Biol.* 10, 866–876.
- (12) Salverda, M. L., Dellus, E., Gorter, F. A., Debets, A. J., van der Oost, J., Hoekstra, R. F., Tawfik, D. S., and de Visser, J. A. (2011) Initial mutations direct alternative pathways of protein evolution. *PLoS Genet.* 7, e1001321.
- (13) Gumulya, Y., Sanchis, J., and Reetz, M. T. (2012) Many pathways in laboratory evolution can lead to improved enzymes: How to escape from local minima. *ChemBioChem* 13, 1060–1066.
- (14) Ghadessy, F. J., Ong, J. L., and Holliger, P. (2001) Directed evolution of polymerase function by compartmentalized self-replication. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4552–4557.
- (15) Barlow, M., and Hall, B. G. (2003) Experimental prediction of the evolution of cefepime resistance from the CMY-2 AmpC β -lactamase. *Genetics* 164, 23–29.
- (16) Cooper, T. F., Rozen, D. E., and Lenski, R. E. (2003) Parallel changes in gene expression after 20,000 generations of evolution in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 100, 1072–1077.
- (17) Meyer, J. R., Dobias, D. T., Weitz, J. S., Barrick, J. E., Quick, R. T., and Lenski, R. E. (2012) Repeatability and contingency in the evolution of a key innovation in phage lambda. *Science* 335, 428–432.
- (18) Elena, S. F., and Lenski, R. E. (2003) Evolution experiments with microorganisms: The dynamics and genetic bases of adaptation. *Nat. Rev. Genet.* 4, 457–469.
- (19) Elena, S. F., Agudelo-Romero, P., Carrasco, P., Codoner, F. M., Martin, S., Torres-Barcelo, C., and Sanjuan, R. (2008) Experimental evolution of plant RNA viruses. *Heredity* 100, 478–483.
- (20) Tenaillon, O., Rodriguez-Verdugo, A., Gaut, R. L., McDonald, P., Bennett, A. F., Long, A. D., and Gaut, B. S. (2012) The molecular diversity of adaptive convergence. *Science* 335, 457–461.
- (21) Elena, S. F., Wilke, C. O., Ofria, C., and Lenski, R. E. (2007) Effects of population size and mutation rate on the evolution of mutational robustness. *Evolution* 61, 666–674.

- (22) Zwart, M. P., Daros, J. A., and Elena, S. F. (2011) One is enough: In vivo effective population size is dose-dependent for a plant RNA virus. *PLoS Pathog.* 7, e1002122.
- (23) Vignuzzi, M., and Andino, R. (2012) Closing the gap: The challenges in converging theoretical, computational, experimental and real-life studies in virus evolution. *Curr. Opin. Virol.* 2, 515–518.
- (24) Woods, R. J., Barrick, J. E., Cooper, T. F., Shrestha, U., Kauth, M. R., and Lenski, R. E. (2011) Second-order selection for evolvability in a large *Escherichia coli* population. *Science* 331, 1433–1436.
- (25) Husimi, Y. (1989) Selection and evolution of bacteriophages in cellstat. *Adv. Biophys.* 25, 1–43.
- (26) Bull, J. J., and Molineux, I. J. (2008) Predicting evolution from genomics: Experimental evolution of bacteriophage T7. *Heredity* 100, 453–463.
- (27) Barrick, J. E., Yu, D. S., Yoon, S. H., Jeong, H., Oh, T. K., Schneider, D., Lenski, R. E., and Kim, J. F. (2009) Genome evolution and adaptation in a long-term experiment with *Escherichia coli*. *Nature* 461, 1243–1247.
- (28) Pande, J., Szewczyk, M. M., and Grover, A. K. (2010) Phage display: Concept, innovations, applications and future. *Biotechnol. Adv.* 28, 849–858.
- (29) Breaker, R. R., and Joyce, G. F. (1994) Emergence of a replicating species from an in vitro RNA evolution reaction. *Proc. Natl. Acad. Sci. U.S.A.* 91, 6093–6097.
- (30) Wright, M. C., and Joyce, G. F. (1997) Continuous in vitro evolution of catalytic function. *Science* 276, 614–617.
- (31) Lincoln, T. A., and Joyce, G. F. (2009) Self-sustained replication of an RNA enzyme. *Science* 323, 1229–1232.
- (32) Szcepanowski, J. T., and Joyce, G. F. (2012) Synthetic evolving systems that implement a user-specified genetic code of arbitrary design. *Chem. Biol.* 19, 1324–1332.
- (33) Esvelt, K. M., Carlson, J. C., and Liu, D. R. (2011) A system for the continuous directed evolution of biomolecules. *Nature* 472, 499–503.
- (34) Bornscheuer, U. T., Altenbuchner, J., and Meyer, H. H. (1998) Directed evolution of an esterase for the stereoselective resolution of a key intermediate in the synthesis of epothilones. *Biotechnol. Bioeng.* 58, 554–559.
- (35) Carr, R., Alexeeva, M., Dawson, M. J., Gotor-Fernandez, V., Humphrey, C. E., and Turner, N. J. (2005) Directed evolution of an amine oxidase for the preparative deracemisation of cyclic secondary amines. *ChemBioChem* 6, 637–639.
- (36) Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., III, and Smith, H. O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* 6, 343–345.
- (37) Summers, W. C., and Siegel, R. B. (1970) Transcription of late phage RNA by T7 RNA polymerase. *Nature* 228, 1160–1162.
- (38) Ikeda, R. A., Chang, L. L., and Warshamana, G. S. (1993) Selection and characterization of a mutant T7 RNA polymerase that recognizes an expanded range of T7 promoter-like sequences. *Biochemistry* 32, 9115–9124.
- (39) Raskin, C. A., Diaz, G. A., and McAllister, W. T. (1993) T7 RNA polymerase mutants with altered promoter specificities. *Proc. Natl. Acad. Sci. U.S.A.* 90, 3147–3151.
- (40) Twigg, A. J., and Sherratt, D. (1980) Trans-complementable copy-number mutants of plasmid ColE1. *Nature* 283, 216–218.
- (41) Stoker, N. G., Fairweather, N. F., and Spratt, B. G. (1982) Versatile low-copy-number plasmid vectors for cloning in *Escherichia coli*. *Gene* 18, 335–341.
- (42) Drake, J. W. (1991) A constant rate of spontaneous mutation in DNA-based microbes. *Proc. Natl. Acad. Sci. U.S.A.* 88, 7160–7164.
- (43) Chelliserrykattil, J., and Ellington, A. D. (2004) Evolution of a T7 RNA polymerase variant that transcribes 2'-O-methyl RNA. *Nat. Biotechnol.* 22, 1155–1160.
- (44) Nei, M., and Chesser, R. K. (1983) Estimation of fixation indices and gene diversities. *Ann. Hum. Genet.* 47, 253–259.
- (45) Holsinger, K. E., and Weir, B. S. (2009) Genetics in geographically structured populations: Defining, estimating and interpreting F(ST). *Nat. Rev. Genet.* 10, 639–650.
- (46) Cheetham, G. M., and Steitz, T. A. (1999) Structure of a transcribing T7 RNA polymerase initiation complex. *Science* 286, 2305–2309.
- (47) Steitz, T. A. (2009) The structural changes of T7 RNA polymerase from transcription initiation to elongation. *Curr. Opin. Struct. Biol.* 19, 683–690.
- (48) Yin, Y. W., and Steitz, T. A. (2002) Structural basis for the transition from initiation to elongation transcription in T7 RNA polymerase. *Science* 298, 1387–1395.
- (49) Poelwijk, F. J., Kiviet, D. J., Weinreich, D. M., and Tans, S. J. (2007) Empirical fitness landscapes reveal accessible evolutionary paths. *Nature* 445, 383–386.
- (50) Tahirov, T. H., Temiakov, D., Anikin, M., Patlan, V., McAllister, W. T., Vassilyev, D. G., and Yokoyama, S. (2002) Structure of a T7 RNA polymerase elongation complex at 2.9 Å resolution. *Nature* 420, 43–50.
- (51) Tokuriki, N., and Tawfik, D. S. (2009) Stability effects of mutations and protein evolvability. *Curr. Opin. Struct. Biol.* 19, 596–604.
- (52) Bloom, J. D., Labthavikul, S. T., Otey, C. R., and Arnold, F. H. (2006) Protein stability promotes evolvability. *Proc. Natl. Acad. Sci. U.S.A.* 103, 5869–5874.
- (53) Drummond, D. A., Iverson, B. L., Georgiou, G., and Arnold, F. H. (2005) Why high-error-rate random mutagenesis libraries are enriched in functional and improved proteins. *J. Mol. Biol.* 350, 806–816.
- (54) Bershtein, S., and Tawfik, D. S. (2008) Ohno's model revisited: Measuring the frequency of potentially adaptive mutations under various mutational drifts. *Mol. Biol. Evol.* 25, 2311–2318.
- (55) Pollock, D. D., Thiltgen, G., and Goldstein, R. A. (2012) Amino acid coevolution induces an evolutionary Stokes shift. *Proc. Natl. Acad. Sci. U.S.A.* 109, E1352–E1359.
- (56) Bershtein, S., Goldin, K., and Tawfik, D. S. (2008) Intense neutral drifts yield robust and evolvable consensus proteins. *J. Mol. Biol.* 379, 1029–1044.
- (57) Wang, X., Minasov, G., and Shoichet, B. K. (2002) Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs. *J. Mol. Biol.* 320, 85–95.
- (58) Jackel, C., Kast, P., and Hilvert, D. (2008) Protein design by directed evolution. *Annu. Rev. Biophys.* 37, 153–173.