

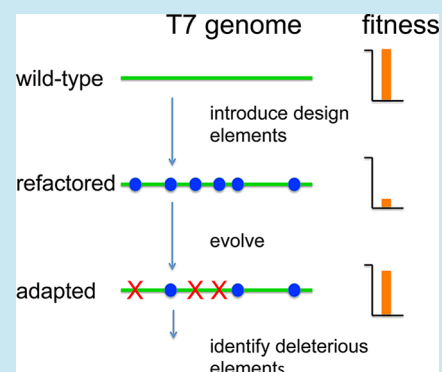
Evolutionary Stability of a Refactored Phage Genome

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S Supporting Information

ABSTRACT: Engineered genetic systems are commonly unstable; if propagated, they evolve to reverse or modify engineered elements because the elements impair fitness. A goal of synthetic biology is thus to anticipate and avoid detrimental engineering, but little is yet known about which types of elements cause problems in different contexts. In prior work, 30% of the genome of bacteriophage T7 was “refactored” by the insertion or modification of 65 short sequences that included a useful restriction enzyme site in order to, among other goals, separate genes and their translational initiation regions from each other and from other genetic elements. Although gene sequences and known important regions of regulatory elements were kept intact, the translational efficiency of some genes or element regulatory function might have been compromised. We adapted the refactored phage for rapid growth in two conditions, observing fitness and sequence evolution. As anticipated from the original work, refactoring had major fitness effects in both environments, but most of the fitness costs were recovered upon adaptation. The evolved phages retained 60–70% of the design elements, suggesting they had only minor fitness effects. Approximately half the elements that were lost lie within large deletions commonly observed during adaptation of the wild-type genome. Some elements were lost or modified in parallel between the adaptations without affecting T7 gene sequences, but no obvious correlates can be made. Nevertheless, experimental adaptations are useful for identifying specific synthetic design problems, and we suggest that experimental evolution in conjunction with alternative engineering may also be useful in overcoming those problems.

KEYWORDS: genome, engineering, experimental evolution, adaptation, design element



Synthetic genetic systems commonly use genetic elements whose functional properties are already known. Modularity of the elements is key to this enterprise, so that elements from diverse genetic backgrounds can be combined with simple linker sequences into interacting networks that create new pathways and functions (e.g., ref 1). It is commonly assumed that the nonfunctional sequences used to join the different molecules are inconsequential. Yet the linker sequences used to join the different modules may have regulatory effects and might impair fitness. Here we consider whether modularizing a genome per se disrupts the patterns of gene expression that are important to fitness.

A model system for evaluating this question is the bacteriophage T7. T7 is one of the best understood of the tailed phages from molecular and physiology perspectives.² It has also been used in studies of in vitro evolution, with characterizations of fitness during adaptation and of molecular evolution across the entire genome accompanying the adaptations;³ T7 has been the subject of an empirically based, kinetic simulation model.⁴ More recently, the first 30% of the T7 genome was refactored, a form of modularization, mostly by insertions of short DNA sequences between genes and between genes and regulatory elements, in part to render the genome amenable to systematic analysis of gene and element function.⁵ The wild-type T7 genome is close-packed, and although only a few coding sequence overlap, many ribosome-binding sites are

in the coding sequence of the upstream gene; such genetic organization may be highly sensitive to precise position effects. However, refactoring did not create, destroy, or otherwise modify elements of known essentiality to T7. It is thus possible to test the fitness effect of refactoring per se, without the complications associated with new combinations of genes and regulators. Plaque size and other simple assays had suggested that refactoring had fitness costs,⁵ but one could not infer just how impaired the engineered genome was, nor whether most or just a few elements were detrimental.

Here we propagate this refactored T7 to enable it to evolve and correct any defects by adaptation. We identify which elements are lost during adaptation and how fitness evolved, in order to assess the implications of genome modularization and which types of modularization are problematic. Last, we suggest how experimental evolution may not only identify design problems but also lead to improved engineering.

The Model System: T7. T7 is a member of the *Podoviridae* that infects Gram-negative enteric bacteria. Its dsDNA genome is ~40 kb, with nearly 60 genes but little coding sequence overlap.² One distinguishing characteristic of T7 is that it encodes a phage-specific RNA polymerase and 17 phage-specific promoters, along with typical DNA metabolism and

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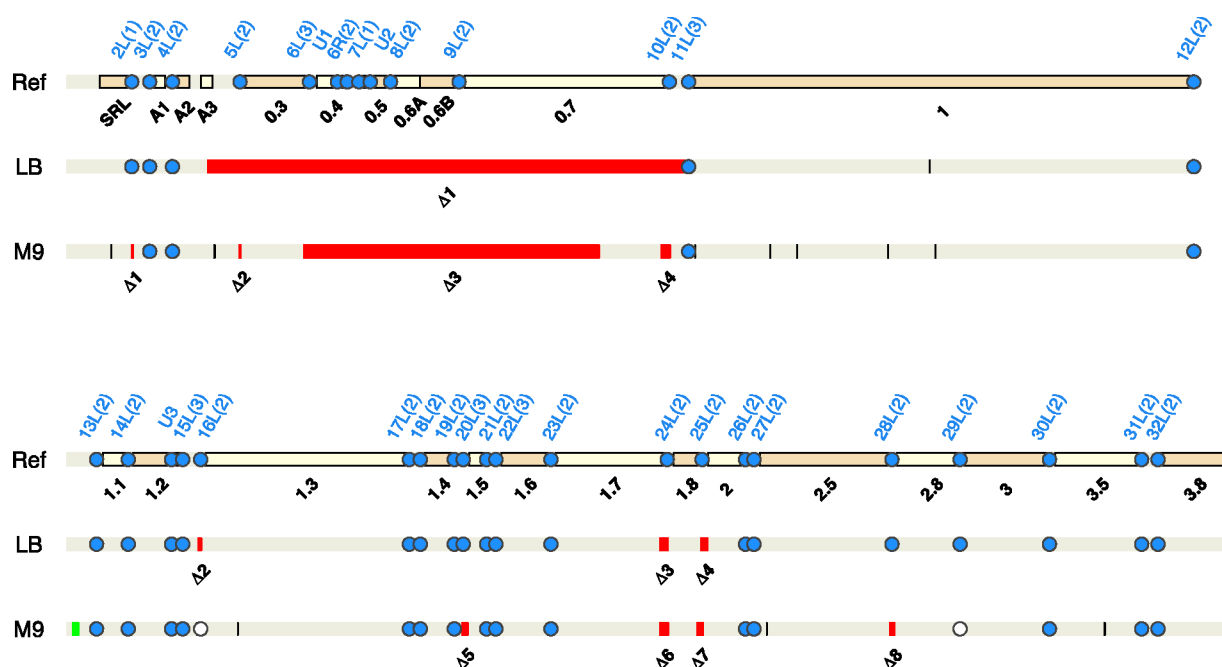


Figure 1. Evolution of design elements in the refactored portion of T7 (α and β sections, extending into gene 3.8). Gene sizes are drawn to scale, so the genome is split near the midpoint of the $\alpha\beta$ region rather than at the junction separating α and β (within gene 1.7). The top genome in each trio is the engineered genome, showing locations of design elements (blue dots), genes, and some regulatory elements (alternating light colors). Design elements from refactoring are labeled above the genome, genes below. The lower two genomes, representing the LB (broth) and M9 adaptations, show the locations of design elements that persisted throughout the adaptation as well as (i) deletions in red (numbered consecutively, spanning the portion of the genome deleted), (ii) substitutions, either as thin, vertical black lines if occurring outside design elements or as white circles if inside the element, and (iii) insertions shown in green. No insertions and only one substitution occurred in the LB adaptation (for this portion of the genome). Two substitutions in the M9 line are obscured by the design elements, one near U3 and one near 15L. Insertion locations are shown without shifting the downstream frame of reference. Most design elements were created in pairs and are too close together for adequate resolution here, so only the rightmost element in each pair is labeled (e.g., the construction of D10R-D11L-gene 1, D11L-D12L is represented in the figure as 11L, gene 1, 12L). Each element label gives the number of elements represented by the label; a “3” is due to unintentional duplication of an element in the design. See Supplementary Tables S1 and S3 for details of these changes as well as changes in the remainder of the genomes.

structural genes. The phage-determined regulation of gene expression provides a basis for understanding and modeling the quantitative dynamics of the intracellular life cycle;⁴ this understanding likewise provides a basis for positioning design elements to avoid disrupting genes and regulatory elements. T7 can evolve to grow extremely rapidly when hosts are maintained in excess and attains a growth rate of 44 doublings per hour, or over 10^{13} descendants per hour in broth at 37 °C.⁶ This high intrinsic fitness enables the virus to grow well even when its genome has suffered numerous insults, either by design or mutation, so it is well suited for genome manipulations. The intrinsic high fitness of T7 also means that slight disruptions of the genome are readily detected and thus it is well suited for identifying potential problems with synthetic designs.

A large number of T7-like genomes have been sequenced; although they can vary extensively at the nucleotide sequence level, they exhibit extensive synteny, varying mainly by the presence or absence of genes with homology to homing endonucleases that have no known role in the T7 life cycle and by the exact complement of non-essential (in the laboratory) early genes. Many recognized regulatory elements are also conserved by genome position in T7-like genomes. Most genes and elements impacted by the refactoring of Chan et al.⁵ fall into the non-essential category: only five of the 21 genes modified are normally considered to be essential. However, the number of progeny per infected cell can be reduced by perhaps an order of magnitude, and fitness of a mutant phage can likely

be reduced (relative to wild-type) by 10–15 doublings/h before plaque morphology differences or titers are readily noted. Thus although any single engineered change may have little effect on the expression of a non-essential gene or function of a dispensable regulatory element, the combination of many changes introduced at the same time may exert a significant disruption in the phage life cycle.

RESULTS

T7 $\alpha\beta$ (the refactored T7) and T7⁺ (wild-type) were each separately adapted for rapid growth under two conditions: using BL21 as host in LB and the K-12 host IJ891 in M9 glucose media. The former condition is collectively referred to as “broth” (subscripted as LB), the latter as “M9” (subscripted as M9). The two broth adaptations consisted of ~100 generations of serial transfer, whereas the M9 adaptations consisted of ~700–1000 generations of growth in a continuous flow system followed by brief adaptations by serial transfer. All environments provided unlimited hosts for the bulk of the adaptation period, so selection favored rapid growth of the phage populations.

The M9 environment was designed after initial results from the T7 $\alpha\beta$ _{LB} broth adaptation revealed a single deletion that included the loss of several early genes (0.3–0.7) and extending ~200 bases upstream of 0.3. Deletions extending into early genes have been commonly observed in prior adaptations in broth of both wild-type and various mutant T7 strains, but never with loss of sequences upstream of 0.3. As the deletion in

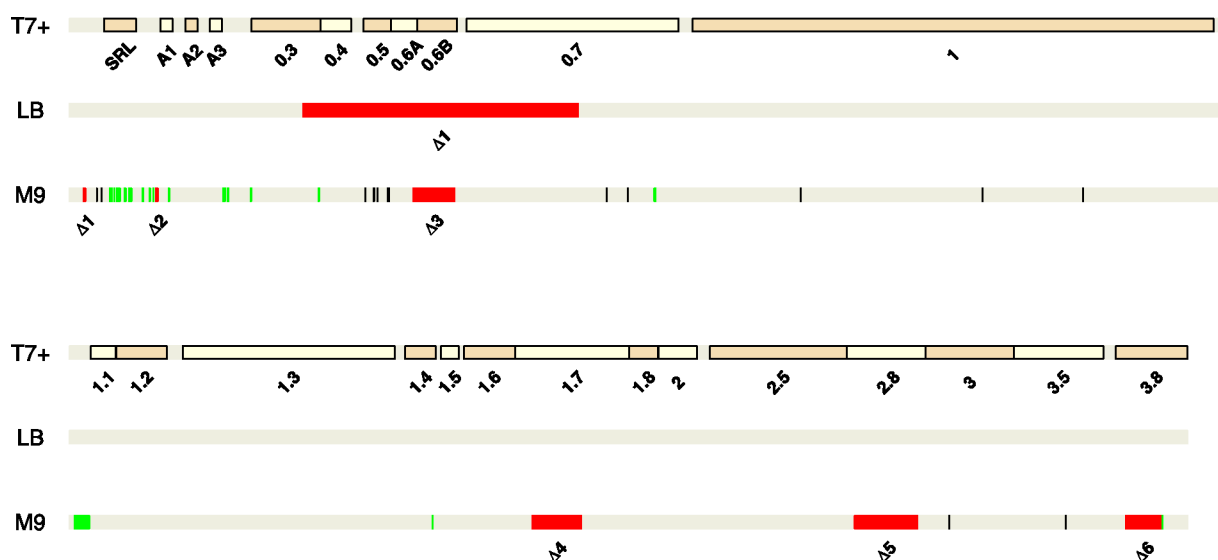


Figure 2. Evolution in control adaptations of the wild-type genome. As in Figure 1, except the phage genomes are the wild-type T7 (top) and the two adaptations initiated with wild-type; only that portion of the wild-type genome overlapping with the refactored region of $T7\alpha\beta$ is shown. Deletions (shown in red in proportion to their size) are numbered consecutively; substitutions are shown as thin black vertical lines; insertions are shown in green in approximate proportion to their size but without displacing the reference frame downstream. The only change observed in the LB adaptation over this portion of the genome was a deletion, but there was profoundly more change in the M9 adaptation. See Supplementary Tables S2 and S4 for details of these changes as well as changes in the remainder of the genomes.

the broth adaptation removed 14 design elements, the intent with the replicate adaptation in M9 was to create an alternative environment that would maintain at least 0.3 and 0.7. We hoped to determine whether their associated design elements were explicitly deleterious or were merely deleted as part of a large region whose net presence was deleterious. M9 media is known to favor retention of 0.7,⁷ and host IJ891 (*hsd⁺*, containing the type I restriction enzyme EcoK1) was expected to select for 0.3, which defends the phage against type I restriction. The M9 environment was thus expected to retain at least 4 and possibly 8 of the 14 elements lost in the LB adaptation of $T7\alpha\beta$.

Molecular Evolution of Design Elements. Both adaptations of $T7\alpha\beta$ resulted in the deletion or modification of several design elements (Figure 1). Sequencing was performed on the evolved populations; changes are reported only if they exceeded 50% as those not reaching 50% would be unlikely confer a significant fitness benefit. Of the original 65 design elements, 40 were maintained after adaptation in M9 glucose and 45 in broth (unintended design element duplications are neglected in these totals, and most design elements were lost as pairs). Despite vastly different levels of molecular evolution between the M9 and broth conditions (Supplementary Tables S1–S4 give all changes in the adaptations, and Figures 1 and 2 show changes in the refactored portion of the genome), both adaptations of $T7\alpha\beta$ resulted in deletions of (i) early region genes and associated design elements (genes 0.3–0.7, with associated losses of 10 design elements in M9 and 14 in broth), (ii) the two elements that separated genes 1.7 and 1.8, and (iii) the two elements between 1.8 and 2. Sequences in (ii) and (iii) evolved back to wild-type. No other design elements were lost or modified in $T7\alpha\beta_{LB}$, but six other design elements were lost or modified in $T7\alpha\beta_{M9}$ (Figure 1). Elements D4R–D5L and D9R–D10L were lost as part of the large deletion of the early region in $T7\alpha\beta_{LB}$ but were lost as separate deletions in $T7\alpha\beta_{M9}$. We can attribute the M9 deletion of D4R–D5L specifically to a fitness cost of

those design elements because no T7 genes or regulatory elements were affected. The M9 deletion of D9R–D10L included the 3' end of 0.7, so the fitness benefit of this deletion need not have been due to loss of the design elements.

Both adaptations to M9 experienced far more and different types of molecular evolution than is typical in T7 adaptations using broth (ref 3, as is also evident when comparing the M9 and broth adaptations here, Figures 1, 2, and Supplementary tables S1–S4). Although the M9 adaptations were done mostly in chemostats and thus for longer than we typically adapt by strict serial transfer, there is no precedent for this much molecular evolution in the many adaptations we have done previously in broth, some of which also used a chemostat. The increased evolution in minimal media was surprising, as minimal media may be imagined to be more typical than broth of the native T7 environment, which is likely sewage. It is noteworthy that the evolved $T7_{M9}$ retained complete 0.3 and 0.7 genes, as designed, whereas $T7\alpha\beta_{M9}$ evolved partial deletions of both. The initial $T7\alpha\beta$ carries a 5-codon deletion in 0.3 (Table S5), but surprisingly, plating assays showed that the gene was functional. Nevertheless, loss of an intact 0.3 presumably imparted some detriment to the phage. The evolved phage retained 4 of 5 Eco KI sites, so the absence of functional 0.3 would have led to a much larger loss of fitness on IJ891. If such a result holds up on further replication, one might speculate that the loss of 0.3 and 0.7 in $T7\alpha\beta_{M9}$ but not in $T7_{M9}$ argues that modularization altered the benefit of those early region genes. However, additional testing is required to rule out that the engineering merely altered the types of deletions most likely to arise, and that the loss of 0.3 and 0.7 in $T7\alpha\beta_{M9}$ in turn merely reflects the first deletion that arose with a net benefit. It is clear that neither $T7\alpha\beta$ gene 0.3 nor 0.7 is required to be intact for growth on IJ891 in M9 glucose media at 37 °C.

Fitness Evolution. Plaque sizes and other assays had indicated that the fitness, especially at 37 °C, of the initial $T7\alpha\beta$ was much reduced from that of wild-type.⁵ The adaptations performed here provide insight into which elements are

problematic and how much fitness is depressed by the remaining elements. Fitness of T7 $\alpha\beta$ was indeed depressed in both environments relative to that of T7⁺ (Figure 3). Despite

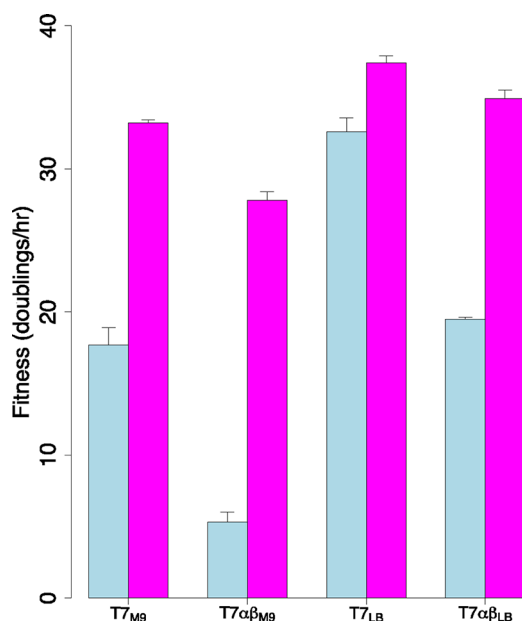


Figure 3. Fitness of wild-type and refactored T7 before (light blue) and after adaptation (magenta). Fitness of the evolved T7⁺ is significantly higher than that of the evolved T7 $\alpha\beta$ in the corresponding growth media (M9: $t_4 = 6.9$, $P = 0.0023$; broth: $t_4 = 5.18$, $P = 0.0066$; significance levels assume a 2-tailed test). Bars represent 1 std error.

considerable fitness improvement, both adaptations of T7 $\alpha\beta$ approached, but did not reach, the fitness of wild-type phage adapted to the same conditions. However, in broth the residual fitness discrepancy is small, suggesting that the remaining design elements have little impact on fitness. Furthermore, our measure of fitness is a growth rate, highly sensitive to small changes in generation time (e.g., lysis time), so this residual fitness impact is possibly due to subtle phenotypic effects. Note that fitness of an evolved T7 $\alpha\beta$ depends not only on residual design elements but also on changes in other parts of the genome, so a difference in fitness between an evolved T7 $\alpha\beta$ and an evolved T7⁺ need not be due to remaining design elements. As one example, T7 $\alpha\beta$ _{M9} is missing large parts of 0.3 and 0.7, whereas T7_{M9} is not, a difference that likely enhances the fitness of T7_{M9}.

DISCUSSION

Extensive refactoring of part of the phage T7 genome created substantial fitness costs. The fact that no genes were eliminated or added in the design suggests that the fitness effects are due to regulatory effects on the phage life cycle. Small changes in the level of transcription or translation of a gene, resulting from synthetically altering intergenic regions, may have large effects on overall phage fitness, especially when fitness is measured as growth rate and is thus sensitive to changes in generation time, as here. Adaptation of the refactored phage in two environments led to substantial fitness increases but fell short of the fitness of T7⁺ adapted to the same conditions. Nevertheless, the majority of design elements remained intact in both adaptations, which means that these changes to the wild-type sequence did not grossly perturb gene expression. This

observation bodes well for the evolutionary stability of *de novo* designs of synthetic regulatory circuitry.

The fitness of T7 when grown under conditions of unlimited hosts is easily depressed by regulatory changes, even seemingly minor ones. When fitness is as high as 40 dbl/h, small changes in lysis time—as little as 0.1 min, only slightly less than 1% of the latent period—have a meaningful effect on fitness,⁶ so the phage is highly sensitive to the duration of its life cycle. It is thus not surprising that the introduction of short segments between genes could impair fitness, but the expectation now is that the effects of most individual insertions should not be large. However, a perspective on the fitness cost of engineering is provided by adaptation of the wild-type genome. The wild-type genome is itself imperfectly “designed”; it invariably evolves/adapts when grown in a constant environment, and the adaptation is usually environment-specific.³ It is thus expected that any form of genetic engineering will also have fitness effects in at least some environments. Indeed, plasticity of a genome enables some level of growth in very different environments where an optimal, specialist genome may not replicate. Where adaptations are particularly informative is in identifying “universal” design flaws, i.e., those elements lost or modified in both environments (parallel changes).

The design elements lost in parallel across these adaptations are too few to draw any strong conclusions about general types of design problems. The deletions identical to both adaptations were of the design elements used to separate overlap between genes 1.7 and 1.8 and a spacer inserted to increase the 2-base gap between 1.8 and 2. Both deletions were facilitated by sequence duplication between the insert and the 3' end of the upstream gene. In contrast, the inserts placed between 1.1 and 1.2, between 1.6 and 1.7, and between 3 and 3.5 (all originally with 1-base overlaps or 1-base separations) had similar characteristics yet remained intact. D15R (shown as 16L in Figure 1) was also lost or modified in both adaptations, yet the intergenic region into which it was placed is relatively large. The attempted separation of the overlap between genes 2.8 and 3 was unsuccessful (Chan et al., 2005), but this overlap is now known to affect the translational coupling of gene 2.8 and gene 3,⁸ and a successful cloning may have been strongly selected against during propagation of T7 β . Interestingly, the $\Delta 5$ deletion that arose in the T7M9 adaptation removes much of gene 2.8 and even causes a frameshift, but the hairpin that sequesters the gene 3 ribosome binding site would still be disrupted as ribosomes translate the mutant 2.8 RNA, thereby allowing translational coupling. The potential importance of sequences outside coding regions cannot be overemphasized when designing synthetic stand-alone genetic elements. Thus, some overlaps and short intergenic regions have substantial regulatory significance, but we cannot yet predict them with confidence.

Deletions of the early region were also observed in both adaptations. The fact that a single, large deletion was observed in the broth adaptation prohibits any conclusion about the fitness effects of the associated design elements *per se*. In the M9 adaptation, however, deletions ($\Delta 1$, $\Delta 2$, and possibly $\Delta 4$) in the early region occurred that were limited to design elements (and perhaps remnants of a deleted gene, in the case of $\Delta 4$), suggesting those elements were specifically detrimental. Even so, because this region is subject to much evolutionary change it is difficult to correlate detrimental effects with specific design elements.

Table 1. Bacteria, Phage, and Plasmids

notation	species/type	genotype	purpose
IJ891	<i>Escherichia coli</i>	K-12 $\Delta lacX74 thi-1 \Delta(mcrC-mrr)102::Tn10$	host used for adaptation in M9 media
BL21	<i>Escherichia coli</i>	B Gal ⁻ <i>ompT hsdS λ^S</i>	host used for adaptation in broth/LB media
T7 $\alpha\beta$	phage	"refactored" T7 genome (Genbank DQ100054, DQ100055 into gene 3.8, wild-type thereafter)	refactored T7 phage used to start adaptations
T7 ⁺	phage	wild-type (Genbank V01146)	wild-type phage used to start adaptations
T7 $\alpha\beta_{M9}$	phage	T7 $\alpha\beta$ with changes evolved for growth on IJ891 in M9 glucose	assess genetic stability of design elements
T7 $\alpha\beta_{LB}$	phage	T7 $\alpha\beta$ with changes evolved for growth on BL21 in broth/LB	assess genetic stability of design elements
T7 _{M9}	phage	T7 ⁺ evolved for growth on IJ891 in M9 glucose	comparison of adaptation between modularized and wild-type genomes
T7 _{LB}	phage	T7 ⁺ evolved for growth on BL21 in broth/LB	comparison of adaptation between modularized and wild-type genomes

Although deletions shorten the genome, allowing it to be replicated faster, and although some deletions may remove coding sequences, lessening the energy cost of translation, it is not obvious that either contributes significantly to T7 fitness. The majority of newly replicated T7 DNA is not packaged into new particles and overall cellular protein synthesis is reduced after T7 infection.² Given the complexities of the adaptation of T7 $\alpha\beta$, perhaps the only accurate way to assess fitness costs of individual elements is to introduce them one at a time into a preadapted wild-type genome. Such an approach is not obviously feasible with so many design elements.

More elements were lost in the M9 than in the broth adaptation. A confounding effect in this comparison is that considerably more evolution occurred outside design elements in M9 than in broth, suggesting that the wild-type T7 genome is not intrinsically as well-suited for growth in M9 as in broth. The large number of changes observed serves as a reminder that attempts to modularize a genome presumes an evolutionarily stable state. Modularization elements are unlikely to be stable if the intrinsic elements of the genome are themselves unstable.

Fitness impairment from refactoring/modularization is not unique to phages. The MIT 'Bibrick' toolkit contains a highly versatile, modularized set of elements for bacterial gene expression, but the elements are intrinsically unstable when bacteria are allowed to adapt.⁹ There are two components to the evolutionary instability: an intrinsic fitness cost and the rate at which mutations arise that improve fitness. Enhanced evolutionary stability was achieved by redesigning the elements to reduce sequences prone to deletion.⁹

Such an approach may be useful for creating a refactored T7 with enhanced evolutionary stability, especially in the design regions expanded by sequence duplication. However, avoiding sequences prone to deletion does not itself overcome the basic problem that design elements may impose a fitness cost. Yet this redesign approach does suggest a stepwise protocol by which genome engineering may be augmented with experimental evolution. The initial adaptation of a synthetic genome will identify problematic elements by their loss or modification. Those elements may then be re-engineered in an informed way into the adapted genome, which still retains many of the original elements. For example, the redesign may avoid sequences most prone to deletion (e.g., by eliminating repeats) but also introduce various types of extra sequences to potentially affect regulation. Subsequent adaptation of the re-engineered genome may then allow evolution to improve the

redesigned elements without removing them, ultimately attaining a fully modularized genome with minimal fitness cost.

METHODS

Strains and media. The bacterial and phage strains are described in Table 1. All are from the collection of I.J.M. or J.J.B. LB broth was 10 g NaCl, 10 g Bacto tryptone, and 5 g Bacto yeast extract per liter. M9 media (6.78 g Na₂HPO₄, 3 g KN₂PO₄, 0.5 g NaCl, 1 g NH₄Cl, 1 mM MgSO₄) was supplemented with 0.2% glucose and 0.01 mM CaCl₂. Plates used LB broth with 1.5% Bacto agar. Determinations of phage titers used plates overlaid with soft agar (0.7% Bacto agar in LB) containing a suitable density of hosts. T7 $\alpha\beta$ and its parent phages T7 α and T7 β were kindly provided by Sri Kosuri and Drew Endy in November, 2005. Titters of the stocks, which were those that had been used by Chan et al.⁴ for sequencing, were very low, but it is not known when the loss of infectivity occurred. Phages were plated on BL21 at 30 °C. All T7 β plaques, but only a minority of T7 α and T7 $\alpha\beta$ plaques contained phages that grew on *E. coli* B (which selects for a functional 0.3 gene). A single plaque of each phage with the desired phenotype was used to provide parental stocks for this study.

Growth of Phage for Adaptation. The purpose of the adaptations was to select rapid phage growth. Adaptation to BL21 in LB broth used serial transfer, as described elsewhere.^{6,10} Briefly, cells from a frozen aliquot were added to 10 mL broth in a 125 mL flask, grown for 1 h to $\sim 10^8$ cells/mL, whence at least 10^5 phage were added. Following phage addition, cultures were grown for 20–60 min (until an adequate density was attained), and then an aliquot added directly to the next flask, whose cells had been grown 1 h. Cultures were sometimes allowed to proceed to lysis, ensuring recombination from high multiplicity infections. T7_{LB} was grown for 21.5 h (80–100 generations), T7 $\alpha\beta_{LB}$ for 26 h (100–120 generations), assuming a 12–15 min generation time.⁶

Adaptations of T7_{M9} and T7 $\alpha\beta_{M9}$ used host IJ891 in M9 glucose media but were each carried out in two stages. The initial stage was growth in a two-stage chemostat, in which actively growing IJ891 in M9 glucose was fed continuously into a tube with phage.¹¹ Phage growth in a chemostat environment can select traits other than fast growth,¹² so a second stage of adaptation was instituted to eliminate any mutations not enhancing growth. Following extensive growth in the chemostat, the final population was recombined against the starting phage, and the recombinant pool was grown by serial transfer.

Serial transfer with IJ891 in M9 glucose used the same methods as with BL21 in LB. T7 $\alpha\beta$ _{M9} was grown for 339 h in a chemostat, then for 7 h by serial transfer following recombination with the initial T7 $\alpha\beta$; T7_{M9} was grown for 226 h in a chemostat, then for 6.5 h by serial transfer following recombination with T7^{*}. Assuming a 20-min generation time in M9, the durations span ~1000 generations and ~700 generations.

Fitness Assays. Fitness was measured in the serial transfer environment, the main difference being that phage densities in fitness assays were maintained at least 10-fold lower than cell density throughout to ensure that hosts were never limiting. All measurements of evolved phages were made on the final populations; those of initial phages were made on lysates made from isolates. Fitnesses were calculated from titers during a continuous serial transfer, 1 and 2 h after phage were added to the first culture to allow attainment of an approximate stable age distribution of infections.⁶ At transfer, a sample from the donor flask was treated with chloroform to stop phage growth, for plating at a later time; in one case of a phage with very low fitness, it was necessary to plate directly from the culture rather than from samples treated with chloroform (used with the initial T7 $\alpha\beta$ in M9). Fitness estimates accounted for any transfer/dilution across multiple cultures during the assay and are presented as doublings per hour, an absolute growth rate that applies regardless of phage generation time.

Sequences. DNA was obtained from phage by phenol extraction and subjected to “454” pyrosequencing at the University of Texas Genomics Core; final populations were used to make the lysates used for DNA extraction. Reads were analyzed by the mapping program *Breseq* (<http://barricklab.org/breseq>¹³). The final, evolved populations were subjected to sequencing, and changes are reported only if they exceeded 50% of the populations, to avoid dealing with sequencing errors associated with the 454 technology; changes not reaching 50% would also not likely have had high fitness. To estimate the extent to which this criterion reflected the magnitudes of molecular evolution in the lines, the evolved T7 $\alpha\beta$ _{LB} population was reanalyzed to provide the quantitative levels of polymorphism. Of the changes shown in Figure 1 (and those given in Supplementary File S3), all but one of the changes were apparently fixed; the deletion starting at 6689 was at 75% frequency. Had the frequency threshold been 20% instead of 50%, only 1 additional change would have been reported in the refactored region (an intergenic substitution between gene *I* and *I.1* at 24%). Thus, the 50% threshold did not give a misleading picture of the evolution.

Our initial isolate of T7 $\alpha\beta$, a clonal population, differed at a few sites from the sequences reported in GenBank (Supplementary Table S5; the sequence of our initial isolate is provided in Supplementary Table S6). Note that the genome reference used in Supplementary Files S1 and S3 use genome positions of our template (Supplementary File S6) instead of the positions from the GenBank entries DQ100054 and DQ100055.

■ ASSOCIATED CONTENT

● Supporting Information

All changes during the 4 adaptations (Tables S1–S4), sequence differences between our isolate of T7 $\alpha\beta$ and the published one (Table S5), and the sequence of our initial T7 $\alpha\beta$ (Table S6). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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