GENOME PROPERTIES AND THE LIMITS OF ADAPTATION IN BACTERIOPHAGES

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Abstract.—Eight bacteriophages were adapted for rapid growth under similar conditions to compare their evolved, endpoint fitnesses. Four pairs of related phages were used, including two RNA phages with small genomes (MS2 and Qβ), two single-stranded DNA phages with small genomes (dX174 and G4), two T-odd phages with medium-sized, double-stranded DNA genomes (T7 and T3), and two T-even phages with large, double-stranded DNA genomes (T6 and RB69). Fitness was measured as absolute growth rate per hour under the same conditions used for adaptation. T7 and T3 achieved the highest fitnesses, able to increase by 13 billionfold and three-quarters billionfold per hour, respectively. In contrast, the RNA phages achieved low fitness maxima, with growth rates approximately 400-fold and 4000-fold per hour. The highest fitness limits were not attributable to high mutation rates or small genome size, even though both traits are expected to enhance adaptation for fast growth. We suggest that major differences in fitness limits stem from different "global" constraints, determined by the organization and composition of the phage genome affecting whether and how it overcomes potentially rate-limiting host processes, such as transcription, translation, and replication. Adsorption rates were also measured on the evolved phages. No consistent pattern of adsorption rate and fitness was observed across the four different types of phages, but within each pair of related phages, higher adsorption was associated with higher fitness. Different adsorption rate limits within pairs may stem from "local" constraints—sequence differences leading to different local optima in the sequence space.

Key words.—Adaptation, bacteriophage, constraint, evolution, experimental evolution, fitness limit, genomes.

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Natural selection has produced many profound adaptations. Scarcely anyone fails to be inspired by the intricate details of camouflage that conceal tasty tropical insects from their predators. Most of us are likewise awed with the speed that pests have evolved to evade the vast array of toxins, drugs, and other chemicals that humans use to control them. The new field of directed evolution has created microcosms of "unnatural" selection to evolve molecules with a diversity of functions from purely random pools of sequence. These and many other examples reveal a seemingly limitless power of selection to create organisms and molecules with characteristics that enhance their survival and reproduction.

Yet there are limits to what natural selection can produce. A billion American chestnut trees succumbed to the Asian blight without evolving resistance (Newhouse 1990). Long-term experimental lineages of Escherichia coli have shown only modest and diminishing increases in fitness (De Visser and Lenski 2002), implying that they are near an upper limit, whereas the fitness gains from short-term drug resistance evolution can be profound. When faced with changing environments, organisms have tracked their old environment more than adapting to the new conditions (Pease et al. 1989), and many species have gone extinct when their habitat disappeared. Organisms have countless dimensions in which to expand their ranges and improve their fitnesses, but the ranges of most species do not appear to be continually expanding (Antonovics 1976). What, then, determines how far an organism adapts?

The magnitude of adaptation that can be achieved is a difficult problem to understand. Evolutionary biologists commonly approach the study of adaptation qualitatively, using evolutionary changes of a particular direction as support for models. Pepper moths evolved to be darker during the days of industrial soot deposition. Body size increases with latitude under Bergmann’s Rule. This qualitative level of analysis is the usual first step when developing and testing a theory. But as evolutionary theories improve, the predicted magnitudes of change become desirable. For example, Ewald (1994) predicted that some of our most virulent pathogens evolved from benign forms (or vice versa) within decades or less. He correctly recognized that the importance of virulence evolution from a social perspective lies in the magnitude of changes in virulence, because slight changes may be inconsequential to public health, whereas large changes can be profound. As another example, the evolution of pesticide resistance by insects and antibiotic resistance by bacteria is a problem only because resistance is so great that the pests are once again able to cause damage in the presence of the chemical. The many biotech applications of evolutionary principles to produce useful molecules depend on obtaining enough improvement in molecular activity to justify marketing.

Understanding limits on the magnitude of evolution for an organism in its natural environment is a daunting task (Antonovics 1976; Barton and Partridge 2000). Trade-offs, plus the vagaries of environmental gradients, spatial variation in population size, gene flow, and competition complicate attempts to measure fitness, rendering it almost impossible to tease apart the factors limiting adaptation. A plant may fail to evolve drought tolerance at the edge of its range because gene flow from wetter areas overwhelms selection for drought tolerance, because the peripheral populations are too small for selection to overcome drift and generate beneficial mutations, or because there are no further mutations to be had.
that improve tolerance of dry conditions without some detriment to other fitness traits.

Many of the problems confronted when observing organisms in the wild can be avoided under laboratory conditions. The growth of a large population within a constant, controlled environment can eliminate the complications of gene flow, spatial variation in selection, interspecific competition, and a plethora of other factors that obtain in the wild. A captive population can be challenged to achieve a specific task (e.g., to survive in an extreme environment, to grow rapidly, or to achieve large body size) and be observed not only for the speed of its response but also for the limit to its response. This method has been used for decades in quantitative genetics to observe the phenotypic response to selection stemming from preexisting variation in the population (Falconer 1989), although the continued change in some long-term lines is thought to be due to new mutations as well (Hill and Bungar 2003). It is now feasible to apply this approach to microbes and molecules and to observe limits to the evolution that comes from new mutations when population sizes are large. Can we then identify rules about the limits of adaptation?

Experimental evolution has commonly been used to adapt viruses and bacteria to artificial conditions (Bennett et al. 1990; Elena et al. 1996; Holland 1996; Travissano and Lenski 1996; Novella et al. 1996; Turner and Chao 1998; Burch and Chao 1999; Holder and Bull 2001; Rokyta et al. 2002). The usual result is that the most rapid improvement occurs early, and fitness eventually reaches a plateau. The existence of a plateau suggests an upper limit to fitness, at least in the short term. The question posed here is whether that limit is determined by general or specific features of the genome.

With this foundation, we offer an experimental protocol to compare the limits of adaptation between eight bacterial viruses (bacteriophages, or phages) adapted to the same environment. Each virus was selected to achieve its maximal fitness (growth rate) in a constant environment. The design here used four pairs of related phages differing in genome size, mutation rate, and genome composition (two phages with small RNA genomes, two with small single-stranded DNA genomes, two with moderate-sized double-stranded DNA genomes, and two with large, double-stranded DNA genomes). By comparing the limits of adaptation within and between pairs of related phages, it should be possible to determine whether genomic factors common to one of the four types of phages ("global" constraints) set fitness limits, or whether limits are set by details unique to each phage ("local" constraints). A precedent for this type of inquiry comes from in vitro studies of ribozyme evolution, in which an RNA ligase incorporating four nucleotides (A, C, G, U) achieved higher fitness/activity than the ligase incorporating only three nucleotides (A, G, U), which achieved higher fitness/activity than the ligase incorporating only two nucleotides (U, and 2,6-diaminopurine; Rogers and Joyce 1999; Reader and Joyce 2002).

**Materials and Methods**

**Strains**

*Escherichia coli* strain IJ1862 was created for this study (*E. coli C/F'128 pifA15 lacZΔM15Tn10*). This strain is able to propagate the eight phages used in this study (Table 1): (1) the single-stranded RNA phages Q8 and MS2 (3–5kb) require the F pilus for adsorption; (2) the single-stranded DNA phages φX174 and G4 (5–6kb genome size) use the *E. coli* C lipopolysaccharide for adsorption; (3) the medium-sized, double-stranded DNA phages T7 and T3 (40kb), of which T7 is intolerant of the pilA product; and (4) the large "T-even" phages T6 and RB69 (170kb). We initially attempted to use the better-known T4 as one T-even phage, but T4 grows poorly on *E. coli* C strains, and we made no attempt to overcome this difficulty.

**Table 1. Genome properties of the eight phages used.**

<table>
<thead>
<tr>
<th>Phage class</th>
<th>Name</th>
<th>Genome composition</th>
<th>Genome size (kb)</th>
<th>No. of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>Q8</td>
<td>ssRNA</td>
<td>4.2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>MS2</td>
<td>ssRNA</td>
<td>3.6</td>
<td>4</td>
</tr>
<tr>
<td>Isometric</td>
<td>φX174</td>
<td>ssDNA</td>
<td>5.4</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>G4</td>
<td>ssDNA</td>
<td>5.5</td>
<td>11</td>
</tr>
<tr>
<td>T-odd</td>
<td>T3</td>
<td>dsDNA</td>
<td>38.2</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>T7</td>
<td>dsDNA</td>
<td>39.9</td>
<td>56</td>
</tr>
<tr>
<td>T-even</td>
<td>RB69</td>
<td>dsDNA</td>
<td>167.5</td>
<td>270</td>
</tr>
<tr>
<td></td>
<td>T6</td>
<td>dsDNA</td>
<td>170(^1)</td>
<td>300(^1)</td>
</tr>
</tbody>
</table>

\(^1\) Numbers are based on the genome of the close relative T4.

General biology of these phages has been described in several edited books: the RNA phages (Zinder 1975), the isometric phages (Denhardt et al. 1978), and all of these phages (Calendar 1988). Genome sequences and genome organizations have been reported for some of these phages as well: MS2 (Fiers et al. 1976), T7 (Dunn and Studier 1983), T3 (Pajunen et al. 2002), RB69 (http://phage.bioc.tulane.edu/html/rb69main.html), φX174 (Sanger et al. 1977), and G4 (Godson et al. 1978). These phages were used from the collection of IJM, except that Q8 was obtained as an infectious clone from D. Mills (Health Science Center, State University of New York, Brooklyn, NY), RB69 as well as T6 were obtained from S. Abedon (Ohio State University, Mansfield, OH), and G4 was ultimately obtained from B. Fane (University of Arizona, Tucson, AZ). The φX174 used to start the selection was an isolate AF from Bull et al. (2000; adapted for rapid growth), and the G4 used to start the selection was an equal mix of three isolates from different stages of adaptation in the study of Holder and Bull (2001). In the latter case, it was thought that the pool of variation would facilitate the adaptation, as 37°C is near the upper end of temperatures tolerated by wild-type G4, and Holder and Bull (2001) had specifically adapted G4 to higher temperatures.

**Adaptation: r-Selection**

Each phage was separately adapted to IJ1862 in a manner slightly modified from that used in previous studies (Bull et al. 2000; Rokyta et al. 2002). Our goal was to select phages with maximal growth rates by growing them in an environment in which hosts were physiologically healthy and more abundant than the phages. This protocol involved serial transfer of phage across multiple cultures of hosts. The basic protocol was to add phage to a 10-ml culture of bacteria at 37°C (± 0.1°C), grown with aeration (200 rpm in an orbital water bath) for 30 to 60 min, then transfer a predetermined
amount directly to another culture. Because the dilution rate exceeded the bacterial growth rate, bacteria could not co-evolve with the phages.

Stocks of JJ1862 were made to facilitate replication of the transfer protocol. Cells were grown in LB broth (10 g NaCl, 10 g Bacto Tryptone, 5 g yeast extract per liter) to a density of approximately 2–4 × 10^8, concentrated 100-fold in LB with 20% glycerol, and frozen in aliquots at −80°C. For passaging, aliquots were thawed, added to 10 ml LB and grown with aeration at 37°C in a 125-ml flask for one hour, such that the density at one hour was approximately 1 × 10^8 (within a factor of 2). Typically, 10^4–10^5 phage were added to the flask and grown until the density reached 10^8–10^9/ml, at which time a sample of 10^4–10^5 phage was transferred directly to the next flask without interruption. After transfer, a sample from the finished culture was treated immediately with chloroform, stored, and later used to establish the titer of phage.

Since phage densities could only be determined by plating, it was desirable to limit each sequence of continuous transfers and to determine phage titers before continuing, to avoid titers too high or too low. The number of consecutive transfers completed without interruption ranged from one to 10, the last culture in the series being treated with chloroform, stored, and used to initiate the next series of continuous transfers at a later time (the number of consecutive transfers was not varied systematically, but was determined largely by convenience). The main drawback of treating a sample with chloroform every transfer is that, when a phage stock is added to a culture, the infection cycles are largely synchronous at first, and chloroform treatment can act to adjust the phage life cycle (lysis time) to match the passage duration. Continuous passaging allows the population of infections to reach a “stable age distribution,” whereby each genotype grows according to its intrinsic rate of increase, regardless of passage duration (Levin et al. 1996). In our experience, asynchrony was dampened within an hour of growth of these phages, but our final steps of adaptation always involved multiple transfers between chloroform treatments to ensure selection for rapid growth.

The interval between transfers was determined by how rapidly the phage titer increased 10^4-fold and ranged from 30 min for the phage with the highest fitness to 60 min for the phage with the lowest fitness; the volume transferred was at least 1 μl and at most 200 μl (from 10 ml of the previous culture). The number of phage introduced at each transfer was chosen as a compromise between avoiding the loss of rare mutations (mutation loss decreases as the number transferred increases) and also preventing phage density from exceeding cell density. If an infected culture progresses to the point that high levels of coinfection occur, selection can be reoriented from rapid growth to competition against other phages in the same cell (Turner and Chao 1998). With this continuous-transfer protocol, one can virtually eliminate bottlenecks by transferring large volumes very frequently, but the practicality of performing these transfers by hand dictates that a 30-min transfer interval is an effective minimum.

Each phage was passaged until fitness had reached an apparent plateau, as indicated by an absence of further fitness increases for at least 10 cycles; in many cases only minor increases were observed over the starting fitness, but passaging was continued far beyond 10 cycles to increase confidence in the limit; one replicate per phage was conducted. The duration of total passaging for these phages were (in hours): QB (70), MS2 (52), φX174 (55), G4 (52), T7 (21), T3 (18), T6 (62), RB69 (47). The generation times of these phages differ: based on published lysis times, they are approximately four generations per hour for T7 and T3, three for φX174 and G4, and one to two for the others. However, generation time evolves as fitness changes (generation time is affected not only by phage metabolism in the cell but also by adsorption rate), so there is no easy way to accurately estimate the number of generations experienced by each phage during the passages. Fortunately, the comparison of fitness and evolution among these phages can be standardized using a scale of absolute time rather than generation time, enabling the comparison of different phages with a single metric (see Results and Discussion for elaboration and justification of this approach).

The protocol was so easily standardized, that, for practical reasons as well as to minimize the chance of contamination, each phage was passaged at a different time from the others. Even so, steps were taken to verify that phages had not become cross-contaminated, using the traits of plaque morphology, PCR, sequencing, and/or host range.

After completion of 32 hours of passaging each, MS2 and φX174 were chosen for further passaging (20 and 23 h) to assess whether the limits we had obtained were perhaps serious underestimates. MS2 was chosen because it had a small RNA genome (expected to have a high mutation rate) yet its fitness limit after 32 hours was one of the lowest obtained; φX174 was chosen because its fitness limit here was below the fitness obtained in another study (under a passaging protocol that involved chloroform treatment between every transfer, Bull et al. 2000).

Assays: Fitness and Adsorption

For ease in comparing results among the different phages, fitness is estimated as the number of doublings of phage concentration per hour. This measure provides an absolute growth rate that is not scaled to generation time (which varies between phages). It represents the absolute capacity of a phage to replicate itself and is proportional to the deterministic growth rate of the phage population on any other fixed interval of time, including generation time. By comparing the doubling rate of different phages on the same scale, it is immediately clear which are the fastest growers. The assessment of fitness was conducted under the same conditions as the adaptation, except that the assays usually used a lower density of phage to facilitate titers with minimal dilutions. At least four separate assays of fitness were performed for all of the adapted lines except for T3 (three measures).

Adsorption assays were conducted by adding 10^6 phage to 10 ml cultures grown under the same conditions used in the adaptations (at least three estimates per phage). At five min after phage addition, an aliquot of the culture was plated immediately to give the concentration of total phage (N_0). A 1-ml sample was centrifuged for 1 min to pellet bacteria and adsorbed phage; an aliquot from the supernatant was then
titered \( (N_u) \). The formula \( N_u = N_0 e^{-kCt} \) (log transformed) was used to estimate \( k \) (the adsorption rate), with \( t \) measured in minutes and \( C = 10^8 \) (cell density). Values of \( k \) were determined for each assay, and statistics were computed on the individual values. Use of frozen aliquots of bacteria allowed standardization of bacterial concentrations between replicates (to approximately \( 10^9/ml \)).

The effect of adsorption rate on phage fitness was modeled as in Wang et al. (1996) and Abedon et al. (2001). Assuming constant host density and metabolic state over sequential episodes of lysis and infection, fitness is proportional to

\[
\int_0^\infty e^{-kCt}B^{(A_t-L)}\ dt
\]

where \( B \) is burst size, \( L \) is the time from adsorption to lysis, \( k \) is adsorption rate, \( C \) is cell density, and \( t \) is the time from release until adsorption (time units are minutes). This model subsumes genome penetration and latent period into \( L \) and treats adsorption probability as an exponential decay process. Equation (1) was computed numerically, given values of \( C, k, B, \) and \( L \).

Statistics

A nested analysis of variance was used to partition the contribution of phage genome type from other factors affecting fitness or adsorption rate. Variance components and significance levels were estimated using formulae for unequal sample sizes (Sokal and Rohlf 1981, p. 294) and with SPSS 11.5 (linear, mixed models, random effects with restricted maximum likelihood estimation; SPSS Inc., Chicago, IL). To test the null model that the ratio of variance components from the nested analysis (between-type/within-type) was the same for two datasets, a simulation was parameterized with the estimates from the data and used to regenerate simulated data with the same sample sizes as in the actual data. The estimated total variance of the original dataset (between-group + within-group) was partitioned in the simulation so that \( p \) of it was assigned to the between-group component, \( 1-p \) to the within-group component. Means for each of the four phage types were drawn using the between-type variance, then a mean for each phage within a type was drawn based on the type mean and the within-type variance. Once an individual phage mean had been set, its fitnesses were drawn with this mean and a variance corresponding to the error variance of the original dataset. Variance components were estimated from the simulated data using the Sokal and Rohlf formulae (1981), and the differences of ratios obtained from hundreds of simulated trials were compared to the differences of ratios in the actual data to assess significance levels.

Tail-Fiber Recombinant T3

An attempt was made to create a recombinant T3 phage with its tail fiber gene (gene 17) replaced by the homologous gene from T7. Gene 17 was cloned from our adapted T7 by PCR amplification and ligation into Invitrogen (Carlsbad, CA) pCR 2.1 vector (the PCR product spanned T7 bases 34569-36534). A plasmid containing the correct insert was identified by PCR, and its ability to recombine with the T3 gene 17 in vivo was confirmed using a T3 gene 17 amber mutant (5% of the recovered phages had lost the amber mutation, several orders of magnitude greater than its reversion frequency). Two methods were used to detect a T7 gene 17 recombinant of our adapted T3. One method scored for the presence of a PCR product (across T7 bases 35001–36312), which should have identified exchanges including the entire 3’ end of gene 17 (but would not identify recombinations that omitted the 3’ end); the 3’ end is thought to confer adsorption specificity. The other method used selective phaging of the T3 recombinant pool on a mutant of E. coli C that does not adsorb T3 but remains sensitive to T7.

RESULTS AND DISCUSSION

Observed Fitness Limits

Estimates of the fitness limits obtained at the ends of the experimental selections showed considerable variation among the eight phages (Fig. 1). The RNA phages and G4 had the lowest limits, whereas the DNA phages with intermediate-sized genomes had the highest limits. In absolute terms, the lowest fitness obtained (phage Qβ, with 8.67 doublings per hour) is a 400-fold increase in phage concentration per hour, whereas the highest fitness obtained (T7, 33.6 doublings per hour) is a 13 billionfold increase per hour. Thus fitness limits varied more than seven orders of magnitude.

This comparison of fitness limits across different phages (with different genomes) is made meaningful because each
phage was adapted to the same environment and fitness was estimated in the selective environment in a way that can be compared across all phages: absolute growth rate. Although there could be some minor differences between our measure of fitness and the actual selection experienced during passages, the estimate of fitness obtained here should closely represent fitness in the passage conditions. The selection favored rapid growth rate under conditions in which hosts were not limiting and multiple infections of the same host were uncommon. In evolutionary ecology, this form of selection is known as r-selection (Pianka 1994). All eight phages were chosen at the outset to be capable of forming plaques on the host at 37°C, so this sample cannot be construed as representative of all phages or even considered a random sample. Rather, phages were chosen so that the selective environment afforded a feasible opportunity for each to adapt through small or large changes. It should be noted that, although the same external environment was used for each phage, the manner in which a phage interacts with that environment is unique to each phage, depending on which host receptors and resources it uses, so the adaptations by different phages are not expected to exploit the same mechanisms or have the same opportunities. Indeed, part of the goal of this study is to understand why some phages are better able than others to exploit this environment.

This protocol was also used to measure the change in fitness that accompanies adaptation: evolvability. Three phages showed large gains during the adaptation (Fig. 1). However, fitness gains here potentially have little evolutionary significance, given the origins and histories of the phage stocks used. All of these phages were isolated from sewage decades ago, and were long maintained as stocks in various laboratories. During that time they were under selection for specific properties that were in some ways different for each phage (e.g., adaptation to a specific host strain) but in some ways similar (e.g., large, uniform plaque morphology). Any fitness improvement seen in these experiments therefore depends on the history of propagation of that phage and cannot be used to reveal any intrinsic biological property of the organism. Indeed, the dx174 and G4 stocks used here had in fact been selected for rapid growth in other studies of ours. The fact that little or no improvement in fitness was observed for a phage may thus mean only that, earlier in its domestication, it had already been adapted to conditions similar to those used here. In contrast, the fitness limit is an absolute value for the conditions employed, which should depend on intrinsic properties of the phage, independently of its recent history. It should be noted, however, that a different set of selective conditions would not only lead to different fitness limits for each phage, but would also likely change the rank ordering between phages.

What Sets the Limits?

Limits to adaptation may stem from an absence of appropriate variation. It is useful, however, to recognize two categories of constraints on variation: global and local. Global constraints are insurmountable because they are imposed by natural laws that cannot be violated, or because a process/molecule used by the phage is encoded by the host and thus cannot evolve through changes in the phage genome. In contrast, local constraints stem from the phage population’s inability to generate certain sequence variants, even though those sequence variants would be beneficial. Small population size, limited mutation rates, and epistasis can all lead to local constraints. Local constraints operate, for example, if the existing sequence is trapped on a local fitness peak and too many mutations are required simultaneously to enter a basin of higher fitness peaks (“absence of fit intermediates” category of Barton and Partridge 2000).

How much of the fitness limit of a phage is determined by global versus local constraints? Our phages differed in genome composition, genome size, genome complexity, and other factors (Table 1). Furthermore, the eight phages comprise four pairs with similar genomic properties: two are single-stranded RNA phages, two are small-genome, isometric single-stranded DNA phages, two are intermediate-genome, and two are large-genome double-stranded DNA phages. Other than this clustering, there is no higher-order relationship evident or recognized, because the genome compositions of the different types of phages are so different as to be incomparable in a phylogenetic context. Global constraints are likely to be similar between related phages but different between phages of different types, and the extent to which global constraints are important in setting fitness limits can be tested with a nested ANOVA. Do phages of similar type have more similar limits than phages of different type? A hierarchical ANOVA (four pairs of related phages) reveals significant variation between the different phage types (variance estimate = 66.0, F1,4 = 10.47, P < 0.025, by the methods of Sokal and Rohlf [1981]; the restricted maximum likelihood estimate is 78.5 with a 95% confidence interval of 13.7–448.4). The ratio of estimated between-type variance to within-type variance is 5.0 (5.8 for the maximum likelihood estimates). There is also significant fitness variation within each pair of phages (P < 10-5 for all pairs except T7/T3, P < 0.26). However, the significance of the between-type effect on fitness can be attributed solely to the high fitnesses of T7 and T3, because the test of the other three pairs of phages alone is not significant (F2,3 = 1.16, P ≈ 0.42).

The global constraint model has some support, but the results are not intuitive. Why should T7 and T3 have superior reproductive rates? Some insight may be offered by considering different types of global constraints. For illustration, we simplify the major aspects of a phage life cycle into a few major processes: adsorption and genome penetration, replication, transcription, translation, viral assembly, and cell lysis. Any of these processes may be rate limiting for phage growth, and selection should then favor mutations that improve this step, except as global constraints prevent evolution. (Similar models have been developed for metabolic pathways, although the processes we define for phage growth are not linearly connected in the same way as steps in simple metabolic pathways; Dykhuisen et al. 1987).

A comparison of these four types of phages with respect to some of the major life cycle processes is given in Table 2. Global constraints operate on those processes for which the phage uses the host machinery. All phages use the host translational apparatus, thus selection for faster translation is limited to optimization of mRNA abundance and increasing
the efficiency of translation of those mRNAs that code for the most abundant proteins, which are predominantly structural. Presumably, the laboratory isolates of these phages are at or close to the optimum for translation, as they have been under intense selection for growth during laboratory passage. Nevertheless, a priori, this constraint should favor the small single-stranded phages where, for example, the number of capsid protein monomers is only 60, but is approximately 420 for T7 and T3 and approximately 960 for T6 and RB69. However, the latter two groups of phages shut off synthesis of host proteins, in part by rapidly inactivating host mRNA synthesis. Competition with host mRNAs for ribosomes is therefore minimized early in the infection cycle. Indeed, the rate of total protein synthesis in T7-infected cells is lower than in the uninfected cell; ribosome availability is therefore unlikely to be limiting. (In contrast, Q8 transcripts initially compete with host transcripts for the ribosomes and only dominate late in the infection cycle; Eigen et al. 1991). Therefore, regarding the global constraint of translation, large-genome phages have the benefit of supressing competition from host messages but have the drawback of requiring more protein per virion than small-genome phages.

An obvious global constraint on replication is genome size: a larger genome requires more precursor (deoxy)nucleoside triphosphate and takes longer to replicate than a short genome. However, T7 and T3 have a major fitness superiority, yet contain much larger genomes than the ssRNA and ssDNA phages. In this case, a major difference in the mechanism of replication between the single-stranded phages and T7 (T3) or T6 (RB69) explains how the latter avoid genome size as their rate-limiting step. Both phage groups degrade the host chromosome, providing large nucleotide precursor pools that need not therefore be imported by the cell or synthesized de novo to sustain replication rates. In addition, both groups code for their own replication enzymes, which are synthesized to higher concentrations relative to the host DNA replication machinery used by the ssDNA phage group. However, in part, the fitness superiority of T7 (T3) over T6 (RB69) may lie in the necessity to replicate about a fourfold larger genome of the latter.

There is also an interesting difference between T7/T3 and the T-even phages that affects the evolution of deletions in the genome. The T-even phages package the same amount of DNA per particle, a “head full,” so that a deletion, which would lessen the replication time of a genome, offers no increase in overall phage reproduction rate. Deletion-mutant particles contain the same amount of DNA as wild-type particles. T7 and T3, in contrast, have fixed ends, so that deletions reduce the amount of DNA per particle.

A second, and perhaps predominant, reason for the superior fitness of T7 (T3) is in transcription. By inhibiting host RNA synthesis, T7 (T3) and T6 (RB69) subsume all cellular ribonucleoside triphosphates to their own ends, whereas the single-stranded phages must share these precursors with host transcriptional demands. The T7 group also codes for a novel phage-specific RNA polymerase that synthesizes RNA five to 10 times faster than the host enzyme. Thus, although T6 (RB69) also directs all cellular transcription capacity to its own genome, transcription of the T7 group genomes is much more rapid.

The fitness difference between φX174 and G4 (19.9, 11.5) is large enough to question whether it is due to local constraints. In an earlier study, fitnesses of evolved G4 and φX174 were similar to each other, approximately equal to that of φX174 fitness obtained here. In addition to treating each passage with chloroform, that study used media supplemented with 2 mM CaCl2 (Holder and Bull 2001). We thus re-estimated fitnesses of G4 and φX174 from this study in media with 2 mM CaCl2. Both fitness averages were close to 20 (data not shown), suggesting that G4 is far more sensitive than is φX174 to low levels of calcium. (From early phage work, calcium is known to strongly affect adsorption rate as well as genome entry rate for many phages; Adams 1959.) The calcium-binding sites of the G4 and φX174 capsids are known from X-ray crystal structures and do not reveal any fundamental difference between the two phages that would suggest differences in global constraints between the phages (McKenna et al. 1992, 1996).

Mutation rates are approximately three orders of magnitude greater for the RNA phages than for the DNA phages (Drake et al. 1998), yet the RNA phages attained the lowest fitness maxima. This result challenges the dogma that high mutation rates lead to high fitness (a dogma also challenged by Sala and Wain-Hobson 2000).

### Table 2. Phage versus host control of phage metabolism.

<table>
<thead>
<tr>
<th>Genome replication</th>
<th>ssDNA phages</th>
<th>T7/T3</th>
<th>T-even phages</th>
</tr>
</thead>
<tbody>
<tr>
<td>one of four polymerase subunits is phage-encoded</td>
<td>uses host DNA polymerase</td>
<td>phage-encoded polymerase</td>
<td>phage-encoded polymerase</td>
</tr>
<tr>
<td>Nucleotide pools</td>
<td>no enhancement</td>
<td>no enhancement</td>
<td>degradation of host genome is catalyzed by a phage gene product</td>
</tr>
<tr>
<td>Transcription</td>
<td>genome is transcript, so replication is equivalent to transcription</td>
<td>uses host RNA polymerase</td>
<td>phage-encoded RNA polymerase</td>
</tr>
<tr>
<td>Translation</td>
<td>no enhancement</td>
<td>no enhancement</td>
<td>host RNA synthesis suppressed, decreasing competition for ribosomes</td>
</tr>
<tr>
<td>Lysis timing</td>
<td>requires cell division</td>
<td>requires cell division</td>
<td>provides precise control</td>
</tr>
</tbody>
</table>
Are the Limits Real?

One obvious concern is that the adaptations were not carried out for sufficient time to observe a true limit—that a larger population size or different spectrum of mutations might have reached an even higher fitness than we observed here (Orr 1998, 2000; Wahl and Gerrish 2001). This concern is mitigated considerably by Fig. 1, which shows that several phages improved only slightly despite extensive passing. For example, the final fitness of MS2 was not even one doubling per hour above its starting fitness, despite 52 hours of passing. After 23 hours of passages, φX174 fitness showed only a slight gain; the phage was then subjected to nitroso-guanidine mutagenesis (20 mg/ml; Hillis et al. 1992) for one passage and then passed for 20 additional hours, yet despite the mutagenesis, the additional fitness gain was only 1.3 doubling per hour.

The attempt here was to identify the limits of adaptation established by constraints on variation and to escape from limits imposed by selective constraints (cf. Barton and Partridge 2000). When a selective constraint exists, population properties prevent the appropriate mutations from occurring or ascending (e.g., small population sizes limit the spectrum of mutations arising and thwart selection through genetic drift, or migration swamps out the beneficial allele in a peripheral population). The protocol here was deliberately standardized to minimize selective constraints, and equally, to ensure that remaining selective constraints were applied across all phages uniformly.

It is obvious that no protocol can escape entirely from selective constraints, because population sizes are invariably finite, and the duration of experimental adaptation is always relatively brief, hence a larger, longer study might yield higher fitness. There are, in fact, empirical indications of the importance of selective constraints in experimental evolution of microbes. Viruses whose fitnesses were deliberately depressed by fixing deleterious mutations have not always recovered original fitness levels when subsequently grown according to a protocol like the one used here (Burch and Chao 1999; Brauer 2000; Bull et al. 2003). Those studies illustrate that a fitness plateau can be reached at a level below that which the virus had attained previously. It is thus important to recognize the nature of selective constraints that apply here, so that their possible importance can be interpreted.

A mutation improving fitness by one doubling per hour requires approximately 20 hours of passing to reach a frequency of 0.5 from an initial frequency of $10^{-6}$ (17 hours if the starting frequency is $10^{-5}$), (based on deterministic equations, Bull et al. 2000). This result holds independently of phage generation time, because the scale is absolute fitness (doublings per hour). Thus this result can be applied to each of the phages used here. For reference to the familiar measure of selection per generation, the selective coefficient of one doubling per hour is simply the generation time of the phage measured in hours, which is at least 0.25 and possibly as high as 0.5 for these different phages. Twenty hours of passing requires considerable effort, yet this effort only ensures the realization of a mutation with a large benefit; separate mutations of smaller effect will thus not likely have time to reach appreciable frequencies in these protocols. There is the further difficulty that, because most of the passages avoided high multiplicities of infection, recombination rates may have been low, and the rapid ascent of mutations of large effect may have depressed the frequencies of previously increasing mutations of lesser benefit (through clonal interference). Hence the limits measured in this study can only be said to apply to fitness improvements that come from mutations of very large effect. Nonetheless, if profiles of adaptation typically show declining rates of fitness improvement with time (e.g., Orr 1998), then the limits reached in this study will reflect the limits achieved in longer-term adaptation to the same conditions.

These considerations suggest that the fitness limit reached by any particular genome should depend not only on genome characteristics (global constraints), but also on local and selective constraints. It is formally impossible to show that a fitness limit reached by a particular genome is in fact due to global constraints, but the use of large population size and long-term selection to achieve an approximate upper limit combined with replication across different phage types adds confidence that the upper limit is due to genome characteristics—the comparative approach. The fact that low fitness limits were observed for both RNA phages (whose genomes differed by more than 30% in sequence) provides a more convincing demonstration that these RNA phages are intrinsically unable to achieve the high fitness levels of T7 than would any reasonable amount of evidence from a single RNA phage. Indeed, if the limits observed here were due largely to the idiosyncrasies of gene sequences unique to each phage, it is surprising that larger differences were not observed within most pairs of related phages. (There were indeed large differences in the fitness maxima between related phages, but except for φX174 and G4, these large differences are nonetheless small relative to the fitness difference between T7 or T3 and the others.) The failure to detect significant heterogeneity among fitness limits of the three pairs of non-T7/T3 phages could in fact be due to local and selective constraints that lead to different fitness limits among related phages. If limits among related phages are not similar, one should use the highest fitness in a group to indicate the upper limit imposed by genome characteristics (in which case T7 and T3 are still vastly superior to the others).

The model of global versus local constraints should be testable in ways that have not been employed here. Specific modifications of the host or the phage may affect fitness limits if those modifications alter global constraints. For example, deletion of the ligase gene from T7 (grown on a ligase-defective host) resulted in a lower fitness limit than when the ligase gene was present (Roktya et al. 2002). Such manipulations, when applied to different phages and replicated adequately to overcome possible local constraints, should give further insight to whether and how genome characteristics affect global constraints and determine fitness limits for different types of phages.

Adsorption as a Model Fitness Component

Insights about fitness limits may be gained by considering a specific fitness component, because a single component should be mechanistically more tractable than is total fitness.
One fitness component with many advantages for this purpose is adsorption rate, which is the rate at which phage attach to bacteria. Higher adsorption should always lead to higher fitness, so there is no optimum (for mixed, liquid environments, whereas an intermediate optimum does exist for growth on plates; Yin and McCaskill 1992). Indeed, very low adsorption rates virtually preclude high phage growth rates (Wang et al. 1996; Abedon et al. 2001). As with fitness evolution, adsorption rate evolution is subject to global and local constraints. The major global constraint is that every phage requires host-encoded receptors for adsorption, the number and accessibility of which are controlled by the host. A host that lacks appropriate receptors is de facto resistant to phages that need those receptors, and one common mechanism by which bacteria evolve resistance to phages is through modification or loss of its phage receptors. Local constraints exist as adaptive valleys and distant peaks in the genotype space of adsorption protein sequences, preventing evolution of sequences with higher adsorption rates. Another advantage of adsorption rate as a tractable model of fitness is that it can evolve independently of other traits. For example, the T phages have tail fibers whose sole function appears to be adsorption. In general, the two RNA phages and ssDNA phages have narrower host ranges than do the T phages, but it is not clear whether the broader host ranges of T phages are due to their tail fibers (host ranges of the RNA phages is potentially broad, but they absolutely require the F pilus for adsorption, and it is only because the F plasmid can infect a broad host range that these RNA phages can also infect various hosts).

Adsorption rate is measured as the coefficient \( k \) in the first-order kinetic model \( dP/dt = -kPC \) (\( P \) is the density of phage, \( C \) the density of bacteria and \( t \) is measured in minutes). The typical range of adsorption rates observed for other phages on susceptible hosts is \( 10^{-8} - 10^{-9} \) (ml/min), and the theoretical maximum is near \( 10^{-8} \) because of limitations imposed by diffusion and the size of bacteria (Adams 1959). Adsorption rates of our adapted phages showed considerable variation (Fig. 2). Except for a nonsignificant difference between MS2 and Qβ, the adsorption rate means are significantly different within each pair of phages (\( P < (2 \times 10^{-6}) \), based on \( t \)-tests), and the nested ANOVA yields a highly significant within-pair component (0.55, \( F_{4,31} = 127 \), \( P = 10^{-12} \)). However, the estimated between-type variance is actually negative, hence not significantly different from zero. (The restricted maximum likelihood procedure could not produce an estimate of the between-type component; the within-type estimate was 0.49, with a 95% confidence interval of 0.17–1.40.) (All tests used \( t(k) \).

The gray curves in the figure illustrate the expected effect of adsorption rate on fitness for different combinations of burst size and latent periods, providing a guide to the pattern expected if all fitness differences were due to adsorption rate variation. There are three noteworthy points: (1) our values lie within the typical range of adsorption rates observed in other phages, with some near the theoretical upper limit; (2) they do not account for most of the observed fitness differences; but (3) within a pair of related phages, the higher adsorption rate is associated with the higher fitness.

An interesting comparison can be made between the variance components underlying fitness versus adsorption rate (suggested by C. Burch, Dept. of Biology, University of North Carolina). The variance due to phage type is a much larger fraction of the total (non-error) variance for fitness than for adsorption rate (83% versus 0%), consistent with the hypothesis that global constraints are a bigger factor in fitness than in adsorption rate. The null model that the two ratios are the same cannot be rejected (simulated datasets show that differences this large can happen more than 5% of the time when the null model is true), but it would be interesting to apply this test to a larger dataset that would provide more statistical power.

A priori, adsorption rate differences between related phages are candidates for local constraints. Consider the differences between T3 and T7 (relative adsorption rates of 0.12 and 0.39, respectively). The tail fiber genes of T7 and T3 are clearly homologous but their products differ in amino acid sequence by 23% (Pajunen et al. 2002), and sequence comparisons among them and other close relatives suggest histories on different hosts that could have trapped the phages on different adaptive peaks. Although the tail fiber proteins of the *Versinia pestis* phage φA1122 and the coliphage T3 are more than 98% identical, the phages do not adsorb to the heterologous host. Furthermore, although expanded host range mutants of φA1122 that grow in *E. coli* are readily found, comparable mutants of T3 are, at best, extremely rare.
(Garcia et al. 2003). On this logic, the adsorption rate of our adapted T3 might be increased (and the local constraint overcome) by replacing its tail fiber gene with the homologue from our adapted T7. Our attempts to generate such a recombinant were unsuccessful, so this possibility remains untested.

CONCLUSIONS

When selected for rapid growth, T7 and T3 achieved much higher fitnesses than the other phages, even though four of the other phages have much smaller genomes, and two also have RNA genomes. This result suggests that the evolution of high fitness is not simply a consequence of small genome size or high mutation rate. We have suggested that fitness limits stem from potential constraints imposed by the host and the manner by which a phage can overcome those constraints.

Adsorption rate, which is expected to experience directional selection, was considerably different between related phages, as if the evolution of this fitness component was more limited by mutation than by genome characteristics. If most fitness components are mutation limited, overall fitness limits could vary greatly even within pairs of related phages, because the factors determining the limit are unique to each phage. We indeed observed considerable variation in fitness limits between related phages, but the overall superiority of T7 and T3 suggests that other genomic properties also contribute greatly to fitness limits.

The mutations that evolve during adaptation will depend both on the mutation rate and on the fitness effect of the mutations. Our passaged introduced bottlenecks, so the only beneficial mutations likely to ascend are those that arise frequently or those with large effects (Otto and Whitlock 1997; Wahl and Gerrish 2001). Thus, the concurrent evolution of different fitness components within a phage may exhibit a limiting similarity: any component far from its maximum will be more likely to improve than a component near its maximum. If genome composition sets an upper limit, then each component may evolve until it is reasonably close to its specific maximum.

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LITERATURE CITED


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