Profiles of Adaptation in Two Similar Viruses

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ABSTRACT

The related bacteriophages ϕX174 and G4 were adapted to the inhibitory temperature of 44° and monitored for nucleotide changes throughout the genome. Phage were evolved by serial transfer at low multiplicity of infection on rapidly dividing bacteria to select genotypes with the fastest rates of reproduction. Both phage showed overall greater fitness effects per substitution during the early stages of adaptation. The fitness of ϕX174 improved from −0.7 to 5.6 doublings of phage concentration per generation. Five missense mutations were observed. The earliest two mutations accounted for 85% of the ultimate fitness gain. In contrast, G4 required adaptation to the intermediate temperature of 41.5° before it could be maintained at 44°. Its fitness at 44° increased from −2.7 to 3.2, nearly the same net gain as in ϕX174, but with three times the opportunity for adaptation. Seventeen mutations were observed in G4: 14 missense, 2 silent, and 1 intergenic. The first 3 missense substitutions accounted for over half the ultimate fitness increase. Although the expected pattern of periodic selective sweeps was the most common one for both phage, some mutations were lost after becoming frequent, and long-term polymorphism was observed. This study provides the greatest detail yet in combining fitness profiles with the underlying pattern of genetic changes, and the results support recent theories on the range of fitness effects of substitutions fixed during adaptation.

ADAPTATION is commonly dissected into the components of selection (differential reproductive success among phenotypes) and the genetic response to that selection. Much of the focus since Darwin has been to understand how differential reproductive success is affected by an organism's environment. This emphasis persists today in the "phenotypic" school of evolution pioneered by Williams, Hamilton, and Maynard Smith and in the quantitative methodology for measuring selection, developed by Lande and Arnold (1983). The genetic basis of adaptation has also been a major focus in evolution, with progress closely paralleling the technologies used in genetic analysis. Heritability estimates, as statistical descriptors of genetics, have been used to predict the response to artificial selection. Methods of biochemical and molecular genetics have more recently helped resolve the functional basis of some phenotypes favored by selection, as in the human sickle-cell trait, self-incompatibility in plants, drug resistance in insects and microbes, and many others (Mitsuhashi 1993; Franklin *et al.* 1995; Golding and Dean 1998; Mazel and Davies 1999; Begun and Whitley 2000).

The genetics of adaptation may be described at another level, one that ignores the functional significance of genetic changes but otherwise monitors the identity and fitness effects of individual substitutions. This approach is similar to the quantitative genetics approach of monitoring the progress of a character in response to artificial selection, but adds the underlying dimension of individual genetic changes during that progress. This description has been the focus of several attempts, new and old, to develop theory for the pattern of fitness change and the distribution of fitness effects per substitution during adaptation (Fisher 1930; Kimura 1983; Orr 1998, 2000). Adaptive processes may exhibit generalities in both the distribution of fitness effects per substitution (Orr 1998, 2000) and in the order of those substitutions through time (Gerrish and Lenski 1998).

This study provides the most detailed analysis yet of genetic changes and associated fitness changes during adaptation. Two bacterial viruses were separately adapted to the same inhibitory environment, the profiles of genome-wide genetic changes and fitness increases were monitored for both, and the data considered in light of the relevant theories. A comparison of fitness changes in the unselected, ancestral environment allows this model system to be viewed as an experiment in viral attenuation, as well.

MATERIALS AND METHODS

Phage and host: The two isometric bacteriophages ϕX174 and G4 were adapted for growth on the bacterial strain *Escherichia coli* C (prototroph) at high temperature. These phage have similar life histories. Upon infection, the single-stranded, circular DNA genome is converted to a double strand by host enzymes. Eleven genes encoded on the genomic strand are expressed by host RNA polymerase. Under ideal conditions,

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obligate lysis with a burst of ~100 progeny follows 15–20 min after infection. These phage do not block superinfection, and recombination mediated by host enzymes can occur in multiply infected hosts (Tessman and Tessman 1959). The 11 genes encoded by ϕX174 and G4 occur in the same genome order and are homologous, but the genome of G4 is slightly larger (5507 bases for G4, 5386 for ϕX174) and the sequences differ by ~33% (Godson *et al.* 1978). The ϕX174 ancestor used in this study was the same one used in Bull *et al.* (1997); its sequence differs at five nucleotide positions from the original sequence of Sanger *et al.* (1977; compare GenBank V01128 to AF176027). The sequence of the ancestor G4 isolate used in this study differs at 20 positions from the published sequence of Godson *et al.* (1978; compare GenBank NC001420 to AF454431).

Selective conditions: The goal was to select the phage in an environment that could be easily replicated in order to ensure that selection conditions could be held constant and that fitness assays accurately reflected the selective conditions. Viral passages were conducted by adding phage to flasks containing rapidly growing cells in Luria broth (LB; 10 g NaCl, 10 g Bacto Tryptone, and 5 g yeast extract/liter), allowing infections to proceed for a limited amount of time (40–90 min), and then transferring phage to a new flask (as in Bull *et al.* 2000). Phage were grown in an excess of bacteria at a low multiplicity of infection (MOI) to minimize intracellular competition, which can lead to frequency-dependent selection (*e.g.*, Turner and Chao 1999). The flasks were incubated in water baths that held temperature to within ±0.1°. Each passage was begun with naïve hosts to prevent co-evolution of the bacteria with the phage. The conditions used for phage transfer (number of phage transferred and duration of phage growth before transfer) were held as constant as possible during the selection, as described below, except that the first few transfers at high temperature often required more heroic efforts to maintain the phage population (larger volumes were transferred and growth time was extended).

ϕX174 passages: ϕX174 was serially propagated at 43.9° for a total of 50 passages (for simplicity in the text below, we refer to this temperature as 44°). Passages were carried out in 1-liter flasks containing 100 ml LB supplemented with 2 mm $CaCl_2$. Aliquots of *E. coli*, from stocks stored at −80°, were thawed and grown in the flasks with shaking until reaching a density of $2–5 \times 10^8$/ml; the time needed to reach that density ranged from 1.5–2 hr. Approximately 10^7 phage were added to the cells and allowed to grow until the population had increased to 10^{10}, a 1000-fold increase in population size. Aliquots were collected and treated with chloroform to kill bacteria. The aliquot was used to initiate the subsequent passage; the remainder was stored in an equal volume of borate EDTA buffer with 14% DMSO at −80°.

G4 passages: Passages were carried out in a manner similar to those of ϕX174, but with smaller flasks (125 ml with 10 ml of LB). Bacteria were grown for 1 hr at the selection temperature to a density of $2–5 \times 10^8$/ml before inoculation with 10^6 phage. Passage duration was calculated to allow a 10,000-fold increase in phage population size.

Wild-type G4 is known to grow at a lower temperature range than ϕX174 (Godson *et al.* 1978). Whereas lab strains of ϕX174 grow well at 37°, lab strain G4 is not obviously well adapted to that temperature. G4 was thus "preadapted" by transferring it through 40 passages at 37° before initiating the high-temperature selection from a single isolate. During the 40 passages at 37°, G4 accumulated two missense substitutions (K380E in gene F and T171A in gene G).

Even after adapting G4 to 37°, the fitness was too low to maintain the phage during serial passage at 44°, so the initial 50 passages were done at 41.5°. A single isolate from the evolved 41.5° population was chosen to extend the selection from passages 51 to 130 at 44°. In addition to this main line of selection, additional experiments were performed with G4, using single isolates or larger samples from different periods of the original line. To facilitate reference to this original line of G4, the notation $G4_t$ will be used to represent the phage from passage t ($t = 0, \ldots, 130$). Thus, $G4_0$ is the isolate from the 37°-adapted line used to start the 41.5° selection, and $G4_{50}$ is the endpoint of the 41.5° selection used to start the 44° selection.

Sequencing and oligo probing: Sequences were obtained from PCR products. The genome was amplified in two overlapping segments, and its sequence was determined from 12 overlapping chain-termination sequencing reactions off the same strand. The primers used for these reactions are as follows, with the nucleotide position indicating the 5′ end, plus or minus indicating which strand (positive or negative) is generated from that primer, and the length of the primer in parentheses: ϕX174 PCR primer pairs 0000+(19), 2953−(17) and 2605+(22), 0058−(20); ϕX174 sequencing primers 0058−(20), 399−(21), 993−(17), 1500− (sequence of this primer is no longer available; this is the approximate location, evident from the sequence generated), 2007−(21), 2536−(31), 2953−(17), 3381−(17), 3849−(16), 4292−(17), 4753−(19), and 5199−(20); G4 PCR primer pairs 5557+(20), 2848−(19) and 2746+(19), 0094−(19); G4 sequencing primers 492+(18), 1039+(18), 1593+(18), 2094+(18), 2610+(18), 2746+(19), 3295+(18), 3782+(18), 4336+(18), 4831+(18), 5317+(18), and 5557+(20). Sequencing reactions were read on an automated sequencer (ABI 377). Sequences were aligned and analyzed in DNASTAR SeqMan II. In some cases, the genomic DNA for the PCR reaction was from an isolate; in other cases, the DNA was from the lysate of a population of phage (referred to as a consensus sequence).

For ϕX174, complete genome sequences were obtained from a consensus of the final, 50th passage, from 5 isolates in this final passage and from an isolate at passage 44; partial sequences were also obtained from another 17 isolates. For G4, sequences were obtained from the isolates that served as the ancestors for the 41.5° and 44° passages, from a consensus of passage 60, from a consensus of passage 80, from 1 isolate at passage 100, and from 5 isolates at passage 130.

Plaque isolates from selected passages were archived in microtiter plates for blotting and analysis by hybridization with radiolabeled oligonucleotides (Crill *et al.* 2000). Oligonucleotides were 17 bases in length with the ninth base complementary to either the ancestral or the derived state of the targeted mutant site. Twelve isolates from every fourth passage of the ϕX174 adaptation and 8 isolates from every third passage of the G4 41.5° adaptation were screened for nucleotide substitutions identified from sequences.

Fitness assays: Fitness assays were carried out according to the protocol of Bull *et al.* (2000) and closely mimicked passage conditions: Cells were grown in 10 ml LB for 1 hr to a density of $\sim 2 \times 10^8$ /ml, 10^2–10^5 phage were added, and the mixture was grown for 40 min. Phage titers of the start and end of the assay were determined by plating. Assays were performed at 37° and 44° for both ϕX174 and G4; additional assays were conducted at 41.5° for G4. Assays were repeated four times for each temperature and phage combination. Fitness was calculated as the $\log_2$ increase in phage concentration per 15 min, as an approximate generation time for these phage. Results of the fitness assays were compared using *t*-tests with Bonferroni corrections. An outlier test was used to remove the value of one fitness assay (Snedecor and Cochran 1980, p. 280), decreasing the variance in the fitness estimate but not qualitatively changing the results.

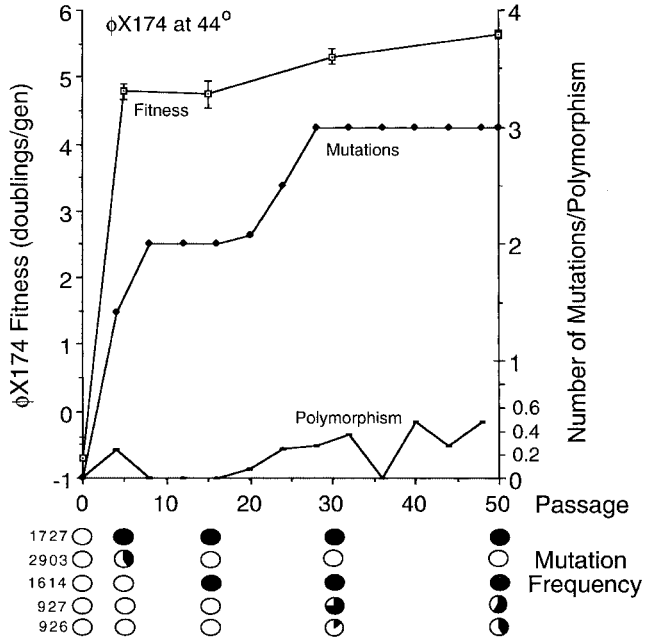

FIGURE 1.—ϕX174 population fitness at the selected temperature, 44°, average number of mutations per genome ("Mutations"), and polymorphism level ("Polymorphism"; $\Sigma p_i(1 - p_i)$ over all assayed sites, where p_i is the frequency of the mutation at that site). Fitness in this and all other graphs is given with error bars indicating ±1 SE. The frequencies of individual mutations are shown below the graph, where white in a circle indicates the proportion of isolates with the ancestral state and black in a circle the proportion with the evolved state. Mutation frequencies are based on oligo hybridization results (12 isolates probed for each time point) for the closest assayed passage (0, 4, 16, 32, and 50). After being detected in sequence data, the changes at 1727, 1614, and 927 were assayed by oligo screening with probes specific to the wild-type and mutant sequences. The change at 2903 was assayed only by sequencing (12 isolates at passage 4, 9 isolates at passage 8, and 5 isolates at passage 50); it was present in only 5 of the passage 4 isolates, so its absence between passages 8 and 50 is inferred. The change at 926 was first detected in the sequence of an isolate at passage 44 that failed to hybridize to the 927T oligo and only weakly hybridized to the wild-type oligo. Of 16 additional isolates in passages 28–50 exhibiting a similar hybridization pattern, sequences of 5 isolates confirmed 926T in all. The presence of 926T in the other 11 is inferred from the hybridization pattern.

RESULTS

Rapid fitness evolution in ϕX174: Figure 1 shows the fitnesses of phage at the indicated time points. From an initial fitness of ~ −0.7, indicating a decline in phage concentration, the population fitness increased to a final fitness of 5.6 at 44°, a net gain of 6.3, or a 40-million-fold increase in the number of descendants/phage/15 min. Most of this increase occurred rapidly, between the second and fifth passage.

Unexpected complexities of molecular evolution in ϕX174: Despite the simple fitness profile, molecular evolution of this phage was moderately complicated (Table 1 and Figure 1). Five mutations were observed in the population during the course of the 50 passages, but only two of them fixed, one was lost, and two remained polymorphic for approximately the latter half of the selection. The mean number of mutations per virus after 50 passages was three. (Generally we reserve the term "substitution" for a mutation that fixed in the population.)

TABLE 1

Nucleotide substitutions accumulated during the high-temperature adaptation of ϕX174

Mutation	Gene	AA change	First detected	Final status
1727 C → T	F242	L → F	4	Fixed
1614 C → A	F204	T → N	8	Fixed
927 G → T	J27	G → V	20	Polymorphic
926 G → T	J27	G → C	28	Polymorphic
2903 G → T	G170	C → F	4	Lost

Figure 1 summarizes the concurrent changes in fitness, mutation frequencies, average number of mutations per genome, and polymorphism across the ϕX174 selection. The first change, detected in passage 2 by a massive increase in fitness, was the substitution 1727T in the major capsid protein gene. This change probably accounted for more than half the ultimate fitness increase, on the basis of estimated fitness of this genotype from another study (BULL *et al.* 2000). The fitness of this genotype was not assayed by itself in this study because at passage 4, the first sampled point, the population contained a high frequency of 2903T in addition to 1727T. The 2903T mutation was not observed at the next sample point (verified by partial sequencing of nine isolates), but by then a second change was present that remained in all subsequent passages (1614A). The other two substitutions, 927T and 926T, both affected amino acid 26 in protein J. 927T was first observed at passage 20, and 926T was observed slightly later at passage 28. By passage 32, all isolates assayed carried one and only one of these substitutions, and both substitutions remained in the population to passage 50 with a severalfold excess of 927T. Assays of individual isolates failed to detect significant fitness differences between the two genotypes (fitness of 926T = 5.4 ± 0.10; fitness of 927T = 5.3 ± 0.15).

G4 fitness evolution was slower than ϕX174 fitness evolution: At the outset, G4 fitness was so low at 44° that the phage could not be maintained at this temperature. The initial passages were therefore conducted at the milder temperature of 41.5°. After 50 passages, fitness at 41.5° had improved from 0.5 to 3.7, for a gain of 3.2 (Figure 2a). Fitness at 44° had also increased enough that the phage could now be maintained at 44°, and the next 80 passages were conducted at this higher temperature. From passage 51 to 130, G4 44° fitness improved from 0.8 to 3.25, for a gain of 2.45 (Figure

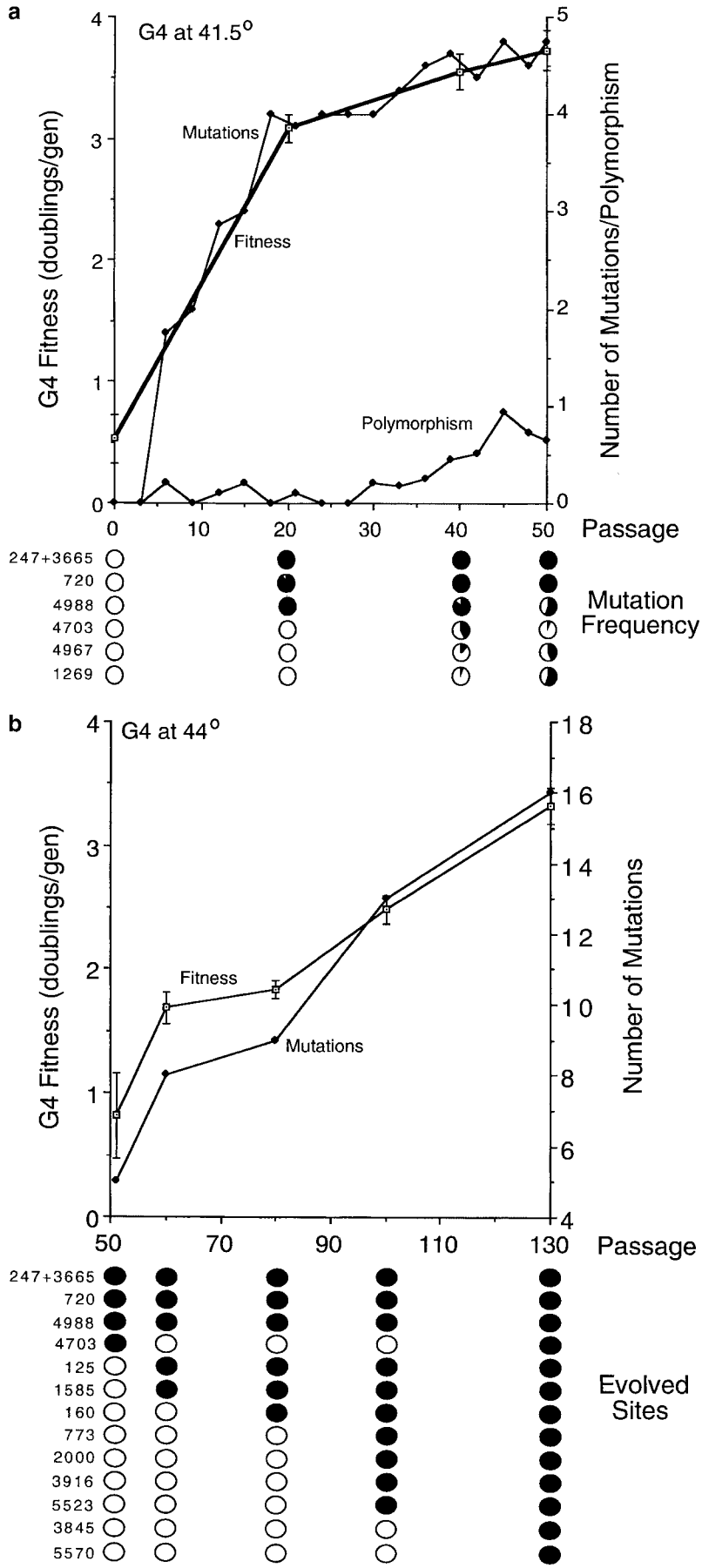

FIGURE 2.—G4 population fitness at the selected temperature with substitution frequencies. White in a circle indicates the proportion of isolates with the ancestral state and black the proportion with the evolved state. (a) Fitness, average number of substitutions ("Mutations"), and polymorphism levels ("Polymorphism") are shown across the entire 50 passages of adaptation at 41.5°. Mutation frequencies are based on oligo hybridization results (8–16 isolates probed for each time point; two time points were combined if they were within 2 of the indicated passage number). The change at 153, found in the sequence of a single isolate from passage 50 is not included because the other isolates have not been probed for the mutation. The Polymorphism curve is $\Sigma p_i(1 - p_i)$ over all assayed sites (see legend to Figure 1). The Mutations curve is the average number of substitutions per isolate at each time point. (b) Fitness and average number of substitutions (Mutations) across the adaptation to 44° (passages 51–130). Presence or absence of a substitution was determined from the sequences. Only five mutations seen in the population at 41.5° were present in the isolate used to initiate the 44° selection. The Mutations curve is the number of substitutions observed in the consensus or isolate sequences at that time point.

TABLE 2

G4 nucleotide substitutions observed after 50 passages of selection at 41.5°

Mutation	Gene	AA change	First detected	Final status[a]
247 C → T	A63	Silent	6	Fixed
3665 C → T	F355	P → S	6	Fixed
4988 G → A	H142	G → D	12	Polymorphic[c]
720 C → T	A221/A*8	T → I	18	Fixed
4703 C → T	H47	A → V	33	Polymorphic[c]
4967 G → A[b]	H135	G → D	15	Polymorphic[d]
1269 A → G[b]	A404/A*191	N → S	42	Polymorphic[e]
153 C → T[b]	A32	S → F	50	Polymorphic

[a] These results are based on sequences and oligo probing. The "Final status" classification is based on probing and on sequences of three isolates at $G4_{50}$ before initiating the 44° selection.

[b] Mutations not present in the isolate used to initiate the 44° selection. After an additional 20 passages at 41.5° (beyond passage 50), the indicated sites were fixed, lost, or remained polymorphic (footnotes *c*, *d*, and *e*). Mutation 153T was not detected in the consensus sequence from passage 70 but was never probed for; mutation 125G was present at 80%.

[c] Fixed at passage 70.

[d] Lost at passage 70.

[e] Remained polymorphic at passage 70.

TABLE 3

G4 nucleotide substitutions observed after selection at 44° (passages 51–130)

Mutation	Gene	AA change	First detected	Final status[a]
125 A → G	A23	T → A	60	Fixed
1585 A → G	B104[b]	T → A	60	Fixed
160 C → T	A34	Silent	80	Fixed
773 G → A	A239/A*27	V → I	100	Fixed
2000 G → T	D9	V → L	100	Fixed
3916 A → G	Intergenic	—	100	Fixed
5523 C → A	H320	N → K	100	Fixed
3845 C → T	F415	H → Y	130	Fixed
5570 C → A	H336	S → Y	130	Fixed

[a] These results are based on consensus and isolate sequences at passages 60, 80, 100, and 130. The status "fixed" means that all five isolates sequenced at passage 130 exhibited the change.

[b] The mutation 1585G is in a region of overlap of genes B, A, and A* but was missense only in B.

2b). Each of these gains is less than the ϕX174 improvement of 6.3 over 50 cycles. Furthermore, the difference in evolutionary rates is even greater than these numbers suggest, because each G4 passage allowed a 10-fold greater population expansion than a ϕX174 passage, so 50 passages of the G4 line were equivalent to 67 ϕX174 passages. Comparing Figures 1 and 2, the sudden fitness increase in ϕX174 at the outset of selection accounts for its greater overall rate, as gains in ϕX174 fitness slowed considerably after passage 4.

G4 molecular evolution and polymorphism at 41.5°: Three substitutions (two missense and one silent) appeared to fix during selection of G4 at 41.5°; at least five additional sites (all missense mutations) were polymorphic in the population at the 50th passage (Table 2 and Figure 2a). The complexities are too numerous to describe in detail, but Figure 2 illustrates fitness, the mean number of substitutions per virus, and the level of variation in the population across the 50 passages. Fitness increases were accompanied by corresponding increases in the mean number of substitutions. Surprisingly, the level of polymorphism also increased across the 50 passages. Recall that both patterns were observed in the ϕX174 profile. Below it is shown that most of this polymorphism in G4 was transient.

Some interesting properties of the evolution are not captured by these statistics. Mutations either fixed rapidly or remained polymorphic for many passages. The two mutations 247T and 3665T were first detected in the same seven isolates at passage 6 and both were present in all later isolates assayed. The mutation 720T was first detected at passage 18 and had completely replaced the ancestral base after passage 21. Although the four other mutations that arose remained polymorphic, two of them (4988A or 4967A) were mutually exclusive of each other and together displaced the purely wild-type genotype (4988A was observed in 90 isolates and 4967A in 14 isolates; isolates wild type for both were not observed in any passage after the first appearance of the earliest of these two mutations). The complete absence of isolates either lacking or carrying both substitutions is significantly nonrandom, but could be ascribed either to epistasis or simply to the fact that they arose in different genomes and were too close to recombine.

G4 molecular evolution at 44°: A single isolate was chosen from the 50th passage at 41.5° ($G4_{50}$) to begin the selection at 44°. That isolate differed from the $G4_0$ ancestor at five sites located in three genes (Tables 2 and 3). Not only did this isolate differ from the consensus sequence in the 50th passage, but also its genotype was not present among the eight isolates probed from $G4_{50}$. From this $G4_{50}$ isolate ancestor, nine nucleotide substitutions, distributed among five genes and an intergenic region, were found in the sequences of each of five isolates from $G4_{130}$ at 44° (Table 3). Of the eight changes located in coding regions, seven resulted in amino acid substitutions. Oligo probing was not used to assay these changes during the 44° selection, so polymorphism statistics are not provided for this part of the selection.

An extension of $G4_{50}$ at 41.5°: Upon completion of the work described above, efforts were directed to understand the nature of the polymorphism at passage 50, the endpoint of the 41.5° selection. An additional 20 passages at 41.5° were carried out, starting from a frozen stock of $G4_{50}$. Ninety-six isolates were assayed from the final passage. Three of the four mutations that were

polymorphic at passage 50 now appeared to be fixed (the derived 4988A and 4703T and the ancestral 4967G; all three were confirmed by a consensus sequence to be a minority type in the thawed $G4_{50}$ stock). Mutation 1269G was still polymorphic, but its frequency had dropped to 0.1. Thus, the level of polymorphism at these sites had decayed substantially. An additional mutation, 125G (seen also in the original 44° adaptation), had evolved to a frequency of 0.8 (Table 2).

Repeatability of evolution during G4 adaptation: The first substitutions to appear during the original 41.5° adaptation were 247T and 3665T, which fixed by passage 9. Selection at 41.5° was continued for another 41 passages during which time an additional mutation (720T) fixed and five others evolved to polymorphism. To test the repeatability of this evolution, part of that selection was replicated by propagating an isolate with 247T and 3665T for 20 passages at 41.5°. Ninety-six isolates were archived from the final passage and probed for three mutations: 720T and 4988A had apparently fixed, and 4703T was present at 2%. For these assayed mutations, this outcome is similar to that of the original adaptation at a comparable time point. By passage 30 of the original line, 720T had apparently fixed and 4988A was at high frequency. The presence of a low frequency of 4703T in the original line can be inferred from its frequency of 0.25 at the next sampled time point.

The original 44° selection was initiated from an isolate of the 41.5° selection (passage $G4_{50}$); by chance, the genotype of that isolate was atypical of the $G4_{50}$ culture. To assess whether a different genetic pathway would have been followed from a different starting point, selection at 44° was repeated in two lines initiated with the polymorphic $G4_{50}$ stock. Consensus sequence of those starting populations exhibited the derived states 247T, 720T, and 3665T that had fixed during adaptation to 41.5°. The consensus sequence also exhibited the changes 1269G and 4967A and the ancestral 153C, 4703C, and 4988G, even though all of these bases were actually polymorphic in $G4_{50}$. Both new lines were propagated for 10 passages at 44°, the only difference being that one line went through a high MOI passage to promote recombination before the 10 serial transfers. The same evolutionary outcomes were observed in both lines after 10 passages: consensus sequences from each line exhibited the derived states 4988A, 1585G, and 125G (plus the original 247T, 720T, and 3665T), and oligo probing of those three changes indicated that they were virtually fixed (of 96 isolates screened from each line, the only ancestral state was 1585A in one isolate). Probing of a fourth mutation, 4703T, indicated that it was absent. Therefore the consensus genotype in these replicates was the same as that observed in $G4_{60}$, 10 passages into the original 44° selection. (In the original 44° selection, 4703T was present in the $G4_{50}$ isolate ancestor but was lost by $G4_{60}$.)

G4 tolerates different substitution orders: Many substitutions occurred across the entire G4 adaptation. Could the order (and identities) of these substitutions have differed? The replicate, short-term adaptations at 41.5° and 44° indicated that the order and identities of changes were substantially repeatable. The similar patterns could have been due to either mutational biases or to epistatic fitness interactions that prevented later mutations from increasing until the earlier ones were established. The existence of such strong epistasis would have important ramifications for patterns of evolution beyond these immediate experiments, so attempts were made to discriminate between the two explanations. Late-appearing substitutions from the 44° selection were introduced at low frequency into populations of $G4_0$, the ancestor used to start the 41.5° selection. These mutagenized populations were subjected to five cycles of selection at 41.5° and then assessed for fitnesses and genetic changes (Figure 3). If those 44° mutations were beneficial only in the presence of the substitutions that had preceded them, they should have disappeared in this selection.

Using PCR products amplified from portions of the $G4_{100}$ and $G4_{130}$ genomes as the source DNA for site-

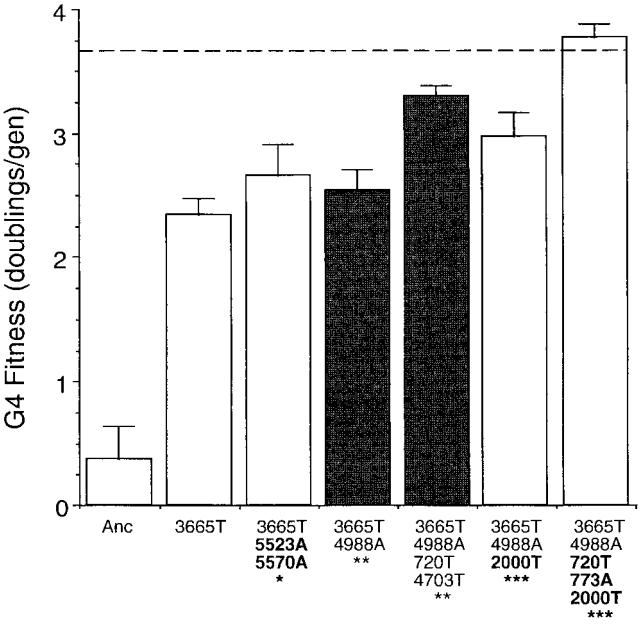

FIGURE 3.—Fitness of G4 genotypes at 41.5°. Open columns are used for fitness of isolates from the site-directed mutagenesis experiments; the first three genotypes are of isolates from one experimental line; the last two are from the other experimental line. Shaded columns are used for isolates from $G4_{12}$ and $G4_{50}$ during the original adaptation to 41.5°. The dashed line indicates the fitness of the polymorphic $G4_{50}$ population. Anc is the $G4_0$ genotype; missense mutations of the other genotypes are listed with site-directed mutations in boldface type. With the exception of the mutation 720T, these site-directed mutations were originally observed toward the end of the 44° selection. Silent mutations: *, 5481C; **, 247T; ***, 1831C and 2818T. They are omitted in the figure to facilitate comparison of the other mutations across genotypes.

directed mutagenesis, 5523A and 5570A were introduced into one $G4_0$ population, and 720T, 773A, and 2000T were introduced into a second population; each population was propagated independently of the other. The frequency of both 5523A and 5570A increased in their line, and 773A, 2000T, and, possibly, 720T increased in their line (data based on oligo screening of isolates and from sequences of two fifth-passage isolates from each line; 720T was not screened but presumably also increased in frequency, because it was observed in the sequences of one of two isolates at the end). In addition to the site-directed mutations, the sequenced isolates exhibited four other changes: two missense mutations observed in the original selection (3665T and 4988A) and three silent changes (Figure 3). The presence of these other substitutions complicates assignment of fitness effects to the site-directed mutations, but it is nonetheless clear that a 41.5° selection could have proceeded by various pathways involving different combinations and orders of mutations observed in the original 44° and 41.5° selections.

Fitness tradeoffs: A tradeoff is manifest as improvements in fitness at one temperature resulting in fitness losses at other temperatures. In these phage adaptations, however, large increases in fitness at the selected temperature did not usually entail a fitness loss at the other temperatures. The 37° fitness of ϕX174 fell significantly below that of the ancestor after 30 passages at 44° (Figure 4a), but the 37° fitness of the evolved G4 was not significantly different from the fitness of its ancestor after a full 130 passages at temperatures of 41.5° and 44° ($P > 0.2$; Figure 4b). During selection of G4 at 41.5°, the gain at 44° actually exceeded the gain at 41.5°. Recall that the site-directed mutagenesis experiments showed that mutations that were beneficial during the selection at 44° were also beneficial at 41.5°.

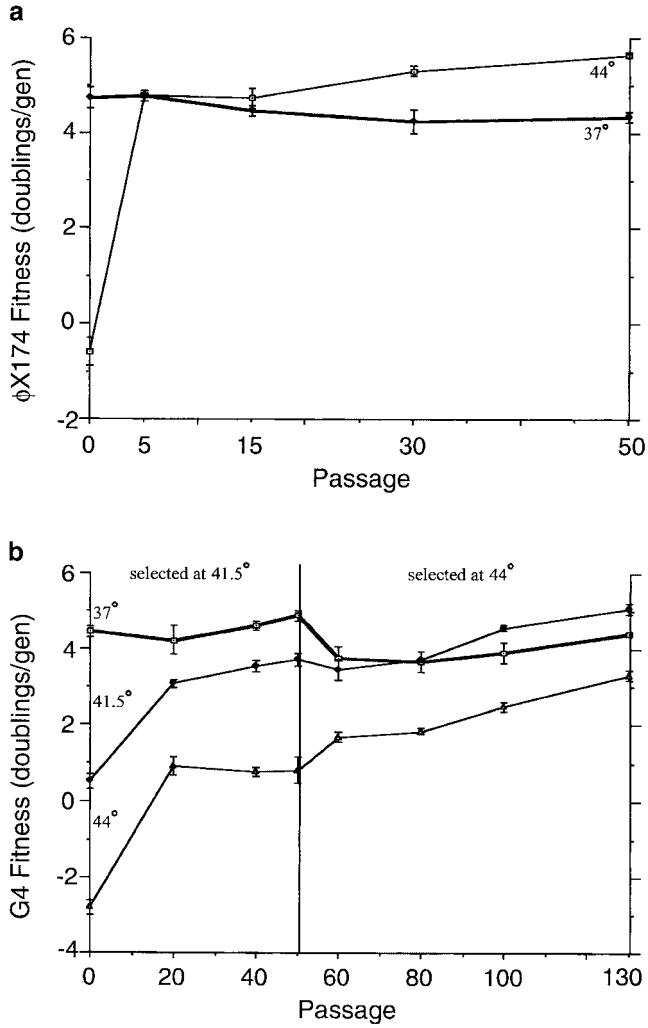

FIGURE 4.—Fitness at multiple temperatures. (a) ϕX174 at 37° and 44°. (b) G4 at 37°, 41.5°, and 44°.

DISCUSSION

This study offers the most precise mapping yet obtained between fitness changes and genome-wide genetic changes during adaptation. The model system used a pair of bacteriophages, ϕX174 and G4, adapted to growth at high temperature on *E. coli* C. Growth at high temperature resulted in large and rapid fitness increases for both phages. Despite the general similarity of the two phages and the equivalent selective environments, their evolutionary trajectories differed in several ways. ϕX174 was adapted directly to 44°. It acquired an average of three substitutions per genome and increased its fitness by 6.3 doublings/generation. G4 was adapted to 44° in two stages. It was initially adapted to 41.5°, where it acquired nearly five substitutions per genome and increased its 41.5° fitness by 3.2 doublings/generation (an increase of 3.6 at 44°). It was then adapted to 44°, where it acquired nine additional substitutions and increased its 44° fitness a further 2.5 doublings/generation. Polymorphism, seen only briefly in the early part of both selections, was more persistent in the latter periods of selection on both phage, although it too was shown to be largely transient at 41.5° in G4 (only the adaptation to 41.5° on G4 was monitored for polymorphism).

This study did not consider the biochemical mechanisms by which these substitutions were beneficial at high temperature (see DOWELL 1980 and BULL *et al.* 2000 for a step in this direction). Even without knowing the underlying biochemical mechanisms, a comparison of the identities of mutations in both phage suggests that the mechanisms of fitness gain were different. The five mutations in ϕX174 occurred in genes F, G, and J; only the two in F fixed. Of the 17 mutations in G4, none were in G or J, and only 2 were in F. The identities of the F mutations differed between the two phage. Indeed, the F amino acid substitution in ϕX174 that conferred the greatest fitness gain was the wild-type G4 residue.

Generalities may underlie the long-term process of adaptation, independent of the functional basis of changes. The description of adaptation at this level is merely one of the order and fitness magnitudes of the genetic changes accumulating over time. Adaptive profiles have recently become the focus of theories attempting to identify statistical properties of the underlying genetic changes (Gerrish and Lenski 1998; Orr 1998, 2000), with roots in the work of Fisher (1930) and Kimura (1983). To the extent that such theories succeed, they might enable one to conduct short-term selection experiments and to predict the ultimate level of progress that could be expected under long-term selection. An understanding of adaptation at this level is certainly relevant to the methods used to develop attenuated viral vaccines, which in the past have been simple, *in vitro* viral passage experiments. One of the outstanding problems with attenuated vaccines is reversion to virulence (Fenner and Cairns 1959). Understanding fitness profiles might help one decide how long an attenuation to carry out and might also help anticipate how many changes would be required to revert the virus to a virulent form. Likewise, knowledge of fitness profile generalities might assist in the development of antimicrobial drugs that are less prone to succumb to microbial drug resistance.

The simplest description of a fitness profile is just the value of fitness measured at different times across the period of adaptation. The limitation of this description is that it confounds the number of adaptive changes with the magnitudes of their effects. Thus, a rapid rise in fitness could stem from one change with a large effect or from many changes with a small effect. If substitutions have measurable benefits and are well spaced in time, the fitness profile alone may be sufficient to identify each genetic change (*e.g.*, Bennett *et al.* 1990; Lenski *et al.* 1991; Elena *et al.* 1996; Burch and Chao 1999). However, if fitness measures are not sufficiently accurate, not sufficiently dense, or if multiple changes arise together, then it will not be possible to identify underlying genetic steps from fitnesses alone. We thus monitored genome-wide genetic and fitness changes during these adaptations. A few other studies have also monitored genome-wide genetic and fitness changes during adaptive processes (Escarmis *et al.* 1999; Wichman *et al.* 1999; Riehle *et al.* 2001). Our study extends that important work in providing a fuller exposition of fitness effects and/or genetic trajectories.

Fitness per substitution: What is the distribution of fitness effects throughout the adaptive process? It is widely accepted (although rarely demonstrated) that, for a novel selective condition, there is often a range of beneficial mutations that can occur, with slightly beneficial mutations outnumbering those of larger benefits (see Burch and Chao 1999 for empirical evidence in favor of this point). Natural selection in turn applies a "filter" to this array of mutations and shifts the distribution toward mutations of larger benefit (Kimura 1983). Theoretical considerations suggest the surprising generality that an adaptive process will fix an approximately exponential distribution of fitness benefits over the course of an adaptive walk of many substitutions, largely independent of the distribution of mutations that arises during the adaptive process (Orr 1998). This result rests on the assumption that adaptation is proceeding toward an optimum, so that the largest possible fitness gains decline as the population continues to adapt. It may not be immediately clear how the concept of an optimum applies to our measure of absolute fitness. However, under constant selection, each viral protein may be viewed as if it has an optimal state in the selective environment, whether its function be enzymatic activity, stability of structure, or other (Golding and Dean 1998). The upper limit of fitness is then itself an optimum at which each protein and other genetic element is at its individual optimum.

One complication in applying this interpretation to some experimental systems (*e.g.*, chemostats) is frequency-dependent selection. In frequency-dependent selection, there is no optimum, because fitness depends on what other individuals in the population are doing, and long-term evolution in such environments often leads to stable polymorphism (Helling *et al.* 1987; Adams *et al.* 1992; Turner *et al.* 1996; Elena *et al.* 1997; Papadopoulos *et al.* 1999). The selective environment used here should have been largely free of frequency dependence, because the average multiplicity of infection throughout the passages was maintained below unity. Selection should thus have placed a premium on rapid growth, with minimal interference from coinfecting viruses.

The model system explored here deviates from Orr's model (which considers an adaptive walk of many steps in which the distribution of fitness effects at each step is predicted by Kimura's 1983 modification of Fisher's 1930 geometric model) in some important ways, and, as a consequence, an exponential distribution of fitness effects is not expected. Our model system differs from Orr's in the following ways:

1. Mutations compete: At the large population sizes employed, different beneficial mutations are introduced simultaneously rather than sequentially. The resulting competition skews the distribution of substitutions in favor of those with large effects (Gerrish and Lenski 1998; Brauer 2000).
2. Mutations are not drawn randomly: At the outset of adaptation, large populations of these phage invariably experience particular mutations known to have extraordinarily large benefits (Bull *et al.* 2000). When combined with point 1, the earliest step of adaptation is nearly assured of being a particular substitution with large effect. In contrast, the random sampling in Orr's model generates a broad distribution of initial fitness effects.
3. Insufficient steps: Too few substitutions occurred per

viral adaptation to adequately approximate an exponential distribution (Orr 1998). The paucity of substitutions might reflect a lack of sufficient time for adaptation, which would be a fault with the experimental design. However, by virtue of the fact that ϕX174 fitness neared what we consider to be an upper limit of fitness (Bull *et al.* 2000), the paucity of substitutions in that phage's adaptation may instead point to a violation of the biology assumed in Orr's model.

Whereas these viral systems may provide an inappropriate match for the strict prediction of an exponential distribution of substitution effects, we can look at a weaker but more robust prediction that the fitness effect per substitution will be greatest during the early phases of adaptation (Gerrish and Lenski 1998; Orr 1998). The main reason for this prediction is that, as the organism approaches its optimum, there is simply less and less room for fitness gain (Orr 1998). A second reason is that, if multiple mutations arise during the same period of time, those with larger effects will ascend the fastest and possibly even displace those of lesser benefit. Thus, if two mutations in different genomes are either incompatible with each other (due to epistasis) or cannot recombine with each other, then the mutation with the largest benefit will prevail (*e.g.*, Gerrish and Lenski 1998 for the case of no recombination). Two factors mitigate against a strict ordering of beneficial effects, however. If beneficial mutations are rare, then they will evolve in the order determined by the mutation supply and escape from stochastic loss; the fact that mutations from late in the 44° selection ascended rapidly when introduced by site-directed mutagenesis to a 41.5° selection suggests that mutation supply was indeed an important determinant of evolution in these experiments. In addition, however, epistasis may operate so that the distribution of fitness effects changes with each substitution: A mutation with no benefit early in the process may convey a large benefit later.

In the experiments reported here, all steps had large effects by standards of natural evolution, but the magnitudes varied. Within each phage, there was a trend that early changes were larger than later ones. The clearest illustration of this pattern was found in ϕX174, with monotonic diminishing returns across three mutations (Figure 5). The first mutation (1727T) likely explains three-fourths of the total fitness gained (on the basis of the fitness estimate from Bull *et al.* 2000), and the second mutation explains a further 15%. In Figure 5, these effects are represented by the slopes of the ϕX174 lines across one, two, and three mutations; although difficult to detect visually, the slope is almost halved between one and two mutations and then declines even further with the third mutation.

The pattern in G4 is less consistent, and aside from a large effect from one of the first two mutations, there

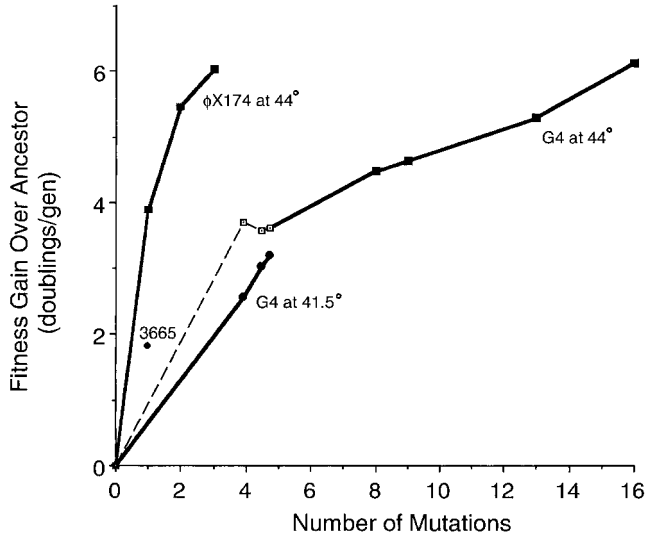

Figure 5.—Population fitness of ϕX174 and G4 as a function of the average number of mutations per genome in the population. The number of mutations were based on oligo hybridization results (ϕX174 and G4 at 41.5°) or consensus sequence (G4 at 44°). For the purposes of this figure, the reversion of the G4 mutation 4703T to the ancestral state and back to the derived state during selection at 44° are counted as separate mutations. This gives 16 mutational events, with a total of 14 substitutions accrued. The dashed line indicates fitness changes in G4 at an unselected temperature. The fitness point for one mutation in ϕX174 is the estimated fitness of 1727T from Bull *et al.* (2000). The point labeled 3665 is the 41.5° fitness of the $G4_0$ ancestor with 3665T from the site-directed mutagenesis experiment.

is no other evidence of diminishing returns. In the G4 selection at 41.5°, an average of almost five mutations accumulated per genome during the 50 passages. One of the first two mutations to fix was 3665T; it was always observed with the silent mutation 247T, so it is not clear if they evolved together or sequentially. On the basis of the fitness estimate from Figure 3, 3665T can account for 57% of the gain at 41.5° from the first 50 passages and thus has a larger effect than later mutations. Figure 5 reproduces this estimate of 3665T as an isolated point, because it is not clear where it belongs on the line. The plot otherwise gives the erroneous impression that the effect per mutation was relatively constant during the 41.5° selection, due in part to the fact that the different points all lie within the range of four to five mutations.

G4 shows a relatively constant effect per mutation across the 11 mutations that occurred during selection at 44°. When the 44° fitness is plotted for the entire 130 passages, the average effect per mutation is greater during selection at 41.5° than at 44°, but as these two selections were performed under different conditions, it is questionable whether this comparison can be used to support diminishing returns under constant selection (the difference could be due solely to the effect of 3665T).

Superficially, the most striking result in Figure 5 is the greater average effect per substitution in ϕX174 than in G4. However, this difference can be attributed to the 1727T mutation, because the other mutations in ϕX174 had fitness effects within the range of those observed in G4 (if the effect of 3665T is included).

The number of beneficial mutations: The models of Fisher, Kimura, and Orr assume an infinite array of possible beneficial mutations. Any generalities expected from those theories will thus be impacted by biological limits to the numbers of beneficial mutations that can occur (Gillespie 1984). Even in those models, however, the chance of experiencing a beneficial mutation declines as the population nears the optimum, so the issue of mutation limitation is perhaps most germane in the early stages of adaptation. Prior studies of ϕX174 adaptation have observed a high level of parallel evolution between replicate lines (Bull *et al.* 1997; Wichman *et al.* 1999; Crill *et al.* 2000). This parallel evolution indicates that the array of highly beneficial changes is limited, but because adaptation will be biased in favor of the best available mutations, there may be a wide array of smaller-benefit mutations that are continually out-competed and thus never seen. In ϕX174 grown at 44°, three different mutations have been isolated that provide enormous benefits as the first step in adapting to this temperature; one of them was the 1727T change observed here (Bull *et al.* 2000). One might suppose, therefore, that an even wider array of beneficial mutations with milder benefits are available, but there is no easy means of assaying that distribution.

It is noteworthy that only 2 of the 22 mutations observed here in ϕX174 and G4 were silent changes within coding regions. The success of one of those silent substitutions (247T in G4) could be attributed to hitchhiking with a missense change, but the other (160T in G4) evolved in the absence of other substitutions and thus must have conveyed a benefit by itself. A benefit for some silent substitutions has been implicated in other studies with these phage on the grounds that the same change has evolved in replicate experiments (Bull *et al.* 1997), whereas hitchhiking of silent changes has been seen as well (Wichman *et al.* 1999).

Selective sweeps: Fisher (1930) and Muller (1932) characterized the evolution of asexual populations as the sequential fixation of beneficial mutations. In their model, a population consisted primarily of a single dominant genotype that served as the background for the next beneficial mutation, which then swept to fixation and became the dominant genotype (the "periodic selection" of Atwood *et al.* 1951). An interesting viral example of this pattern may be seen in the worldwide population of some influenza subtypes. Within a single hemagglutinin subtype, there is a modest amount of variation at any point in time, but that variation all stems from a recent common ancestor, as though the population is frequently purged by one of the variants, which then gives rise to its own set of variants (Buonaguro *et al.* 1986; Bush *et al.* 1999). Periodic sweeps of substitutions also characterized populations of ϕX174 in chemostats (Wichman *et al.* 1999).

Selective sweeps characterized the adaptive profiles in this study, except for some substitutions that disappeared after being detected and some long-term polymorphisms. In G4 (at 41.5°) the polymorphism was shown to be transient, suggesting that it resulted from the fortuitous co-ascent of mutations with similar fitness effects. Those polymorphisms thus represent slow selective sweeps. The same explanation could underlie the long-term polymorphism in ϕX174, although the long-term resolution was not studied directly. It is puzzling that different mutations with such similar fitness effects would exist in these viruses with such small genomes.

The fact that some mutations were lost after ascending to moderate frequencies suggests either of two mechanisms: epistasis or clonal interference. In this context, epistasis is manifested as a mutation beneficial in one genetic background but detrimental in another. Thus if mutations A and B are each beneficial alone but detrimental together, both may initially ascend, but only one will prevail. Clonal interference is instead a competition between compatible, beneficial mutations that happened to have arisen in different, nonrecombining genomes and that are doomed to interfere with each other until one wins (Muller 1932; Hill and Robertson 1966; Gerrish and Lenski 1998). There is evidence both for and against epistasis as a cause of interference in our study. In favor of epistasis we have the following: First, recombination is known in isometric phage (Tessman and Tessman 1959), which mitigates against clonal interference. Second, the 4703T mutation was fixed in the G4 lineage that initiated the 44° selection, yet it was lost and then re-evolved. It is difficult to reconcile this result with clonal interference, as it requires that another beneficial mutation arose in a (rare) genome that had reverted to 4703C. Third, the changes 926T and 927T evolved to polymorphism in ϕX174. Although recombination between these two adjacent bases would have been unlikely, both changes affected the same amino acid, and combining them into the same genome would have produced an amino acid that differed from either single substitution. This evidence merely suggests that epistasis, rather than clonal interference, explains the loss of some changes and polymorphism of others. In contrast, the site-directed mutagenesis experiments in G4 did not support strong epistasis, because substitutions that were observed near the end of selection at 44° were also beneficial in the absence of many of the earlier substitutions. Of course, it may be that strong epistasis accounts for some but not all of the observed interference. Final resolution will require competition studies with site-directed mutants of the recombinant genotypes.

Tradeoff: An assumed and often demonstrated generality of adaptive evolution is a tradeoff: An increase in

fitness in one environment comes at a fitness cost in other environments (Huey and Hertz 1984; Bell 1997). Tradeoffs underlie the rationale behind making viral strains avirulent by growing the viruses in novel environments (Bull 1994). Tradeoffs also motivate the hope that antibiotic resistance in bacteria will wane upon cessation of antibiotic use (Schrag and Perrot 1996). However, most steps in our viral adaptations did not obey tradeoffs: Most of the changes evolved at elevated temperatures were not accompanied by fitness losses at other temperatures. Had these viral adaptations to 44° been attempts to attenuate the viruses at 37°, the attenuations would have failed despite the considerable adaptation to 44°.

An explanation for these results that does not violate tradeoff concepts is that our viruses were not fully adapted to 37° culture conditions before elevating the temperature. Tradeoffs apply strictly to the boundaries of fitness surfaces, and evolution from a point inside the surface need not obey a tradeoff. Changes that evolved at a higher temperature may thus have improved adaptation to the culture conditions over a range of temperatures. Two observations support this interpretation: (i) ϕX174 adapted to 37° in Bull *et al.* (2000) had a higher 37° fitness than the initial phage isolate used here, and (ii) the early G4 adaptation to 41.5° improved fitness at 37°. The fact that G4 adaptation to 41.5° also improved fitness at 44° is a further illustration of the principle that fitness effects can be positively correlated across different environments when a population is not fully adapted to either environment. Stated another way, selection for growth at 41.5° constituted a weak form of selection for growth at 44° (an illustration of Wallace's 1989 stepping-stone model of adaptation).

A failure to observe tradeoffs has precedents. In studies of high-temperature adaptation of *E. coli* already well adapted to culture conditions, correlated changes at nonselected temperatures were found to be mostly positive (Bennett *et al.* 1990, 1992). Likewise, attempts to attenuate virus virulence on its natural host by adaptation to culture conditions has not always succeeded (Fenner and Cairns 1959). With antibiotic resistance in bacteria, while there may be an initial cost to resistance, the cost may be eliminated by subsequent compensatory mutations (Schrag and Perrot 1996; Lenski 1998).

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