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Lysis Time, Optimality, and the Genetics of Evolution in a T7 Phage Model System.

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Lysis Time, Optimality, and the Genetics of Evolution in a T7 Phage Model System.

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Dedication

This is for T7 bacteriophage. You may not have evolved to a quantitative optimum, but no matter how many times I kicked you, you kept standing right back up.

Acknowledgements

I do not and could not thank my parents, Lucinda and Richard Heineman, enough for all they have done for me. They have always encouraged and supported me in whatever I tried, and put up with an incessant stream of snark that could devastate a lesser sincerity than theirs. They are, in both senses of the word, awesome.

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Lysis Time, Optimality, and the Genetics of Evolution in a T7 Phage Model System.

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The ability of traits to adapt in response to change is one of the most fundamental aspects of evolution. Optimality models used to predict adaptation frequently make simplifying assumptions about the ability of traits to evolve freely within simple trade-offs. However, we frequently have little understanding of genomic mechanisms underlying phenotypic evolution. Genetic constraints clearly limit phenotypic change, but the extent to which they do so is unclear. I will explore molecular and phenotypic responses to genomic and environmental perturbations through experimental evolution in T7 bacteriophage.

First, I studied evolutionary robustness of the lysis time phenotype when lysin gene *lysozyme* was deleted. This deletion profoundly delayed lysis and thus decreased fitness. Evolved phages recovered much of the lost fitness and mostly restored lysis

timing. The recovery was mediated by changes in a tail fiber gene (gene *16*) with muralytic activity that is generally used in genome entry.

Next, I extended the work on *lysozyme* to observe the effect of increasing constraint on evolutionary recovery. The effects of various combinations of deletions of *lysozyme*, *17.5* (which plays a role in lysis) and *16* suggested that another gene played a role similar to *17.5* in lysis. The phage defective in both *lysozyme* and *16* did not lyse hosts thoroughly even after long periods of infection, suggesting that these were the only effective lysin genes. Adaptation of this phage on cells expressing the essential gp16 constrained the primary adaptive pathway of recovery from lysozyme deletion. A mutually exclusive alternative pathway involving a variety of different genes evolved. The line recovered the ability to lyse normal hosts, by a mechanism involving multiple mutations.

Finally, I tested the ability of T7 to adapt to an optimum lysis time. Based on empirical results from other phages, mature phage virions accumulate linearly inside the cell over time. This assumption underlies a model suggesting that availability of hosts determines optimal lysis time. While adaptation to different host densities caused the expected qualitative evolutionary changes, adaptation to conditions expected to select for slow lysis did not lead to the quantitative optimum. This is probably due to nonlinear virion accumulation.

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Chapter 1. Background.

Phenotypic traits are formed by the interaction of genetic mechanisms and selective pressures. Optimality models generally assume that traits evolve freely in response to selection within simple trade-offs. But does this approach succeed in predicting evolution, or must genetic details be considered? These questions are most easily answered in a system where selective pressures can be very well understood and controlled for, and where evolution at the genetic and phenotypic level can be explicitly tied to environmental selection pressures.

Experimental evolution is made possible by the large population sizes and short generation times of bacteriophages, viruses that infect bacteria. This method allows us to pinpoint adaptive selection in a way that is impossible in most systems. By looking at both the effect of individual mutations and the overall genetic change, we combine the best of genetics and genomics.

One powerful framework to study the genotypic nature of phenotypic adaptation is lysis time in bacteriophages. It is controlled by only a relatively small number of genes in most phages and a general molecular model has been well-supported in many phages (Young 1992). Lysis time is easily measured and highly variable even between phages with minor genetic differences (Wang 2006). Moreover, an optimality model suggests that lysis timing can be predicted accurately without taking genetic details into account (Wang et al. 1996). The fact that this optimum is determined by conditions likely to be highly variable in natural conditions suggests the trait is likely to frequently be under selection to change in nature. These conditions are also easily manipulated in the

laboratory, so that we can select for both slower and faster lysis. By doing so, we can determine the evolvability of the trait. Since a quantitative version of the model exists (Bull 2006), it is possible to determine whether lysis evolves *to* a predicted optimum rather than merely if it evolves *towards* the prediction. The combination of quantitative predictions and experimental evolution is a powerful approach to studying optimality that has rarely been employed.

Lysis time in T7 is particularly interesting because it does not seem to precisely fit the general model of bacteriophage lysis. As such, the studies here are informational about the genes involved in lysis itself as well as the genes involved in evolution of the trait. The complexity and redundancy of T7 lysis may make it difficult to fully understand the genetic basis of lysis without following a method similar to that used here, determining candidate genes as those which evolve when the primary gene is deleted or when the phage is put under new selective pressures.

This method also has the advantage of allowing us to understand the evolutionary redundancy of lysis genes. Complete genomes may evolve relatively easily to new conditions due to past selective pressure for adaptable lysis. What happens as we reduce the evolutionary toolkit of T7 by deleting or constraining lysis genes? Does the phage still overcome molecular details and re-acquire a close to optimal phenotype? Is the new genetic basis of the trait far less efficient than the old one?

In brief, this dissertation indicates that genetic details are very important to precise control of phenotypes, but that even when many genes are constrained phenotypes evolve qualitatively as expected. Also, control of lysis time is far more complex in T7 than the simple model of bacteriophage lysis suggests.

Chapter 2. Evolutionary robustness of an optimal phenotype: re-evolution of lysis in a bacteriophage deleted for its lysin gene.

ABSTRACT

Optimality models are frequently used to create expectations about phenotypic evolution based on the fittest possible phenotype. However, they often ignore genetic details, which could confound these expectations. We experimentally analyzed the ability of organisms to evolve towards an optimum in an experimentally tractable system, lysis time in bacteriophage T7. T7 lysozyme helps lyse the host cell by degrading its cell wall at the end of infection, allowing viral escape to infect new hosts. Artificial deletion of lysozyme greatly reduced fitness and delayed lysis, but after evolution both phenotypes approached wild-type values. Phages with a lysis-deficient lysozyme evolved similarly. Several mutations were involved in adaptation, but most of the change in lysis timing and fitness increase was mediated by changes in gene *16*, an internal virion protein not formerly considered to play a role in lysis. Its muralytic domain, which normally aids genome entry through the cell wall, evolved to cause phage release. Theoretical models suggest there is an optimal lysis time and that lysis earlier or later than this time decreases fitness. Artificially constructed lines with very rapid lysis had lower fitness than wild-type T7, in accordance with the model. However, while a slow-lysing line also had lower fitness than wild-type, this low fitness resulted at least partly from genetic details which violated model assumptions.

INTRODUCTION

A large body of work in evolutionary biology addresses the adaptive value of phenotypes, such as life history and behavioral traits, in the context of ecology (Charnov 1982; Freeland et al. 2000; Smith 1983; Trivers 1983; Williams 1966). By necessity, genetics of phenotype are often ignored in these approaches, except to posit trade-off functions that establish boundaries on the set of possible phenotypes. These trade-offs often suggest optima, maximally adaptive phenotypic values under particular conditions. A potential limitation of this approach is that the genetic system producing a phenotype may constrain its evolution in ways not captured by the trade-off, thus preventing attainment of the optimum or directing evolution toward pathways not predicted by the purely phenotypic model (Lewontin 1989). For example, models of optimal behavior might fail if it is impossible to evolve to make a particular decision. The reliance on purely phenotypic models is often a necessity, because the genetic nature of phenotypes is almost always unknown, but the rapidly advancing science of genomics may allow us to accommodate their genetic bases. A precedent for this marriage of phenotype with genetic details already exists in bacteriophage lysis. In this study, we examine the genetic basis of lysis recovery to an optimum.

Lysis, a violent rupture of the bacterial cell, is the means by which most bacteriophages cause the release of their progeny from the bacterial host. The timing of lysis is a major fitness component for a phage. It may be considered equivalent to the age of maturity for organisms that die after their first reproduction, such as salmon and century plants. Early lysis of a phage-infected cell has the drawback of releasing few progeny but the benefit of a short generation time, the latter being especially

advantageous when hosts are abundant. Conversely, late lysis allows phage to take better advantage of a particular host by producing more progeny, an advantage when hosts are scarce. There is thus an optimal lysis time which varies with host density and host physiology (Abedon et al. 2001; Abedon et al. 2003; Wang et al. 1996; Wang 2006), and this dependence of the optimum on ecological variables means that lysis time is likely to evolve in nature in response to changing environmental variables.

The genetic basis of lysis of most known phages is relatively simple. All phages containing dsDNA have some form of endolysin gene which provides the critical lysis function - an enzyme with muralytic activity that degrades the peptidoglycan/murein cell wall but which, by itself, cannot access the cell wall. Therefore, most of these phages have additional genes, holins, thought to control lysis timing. Holins permeabilize the inner membrane, allowing endolysins access to the cell wall at the appropriate time (Young 1992). Thus, lysis offers the unique combination of a phenotype that can be addressed from an ecological optimality perspective and whose genetic basis is known.

If lysis is generally controlled by relatively few genes, as is currently thought, how does a phage evolve in response to elimination of its endolysin gene? In particular, will the phage be permanently debilitated or will it reinvent a mechanism of lysis through compensatory evolution, and if so, how many and which genes will be involved? From the optimality perspective, will the newly evolved lysis phenotype be similar to or as efficient as the original, and does the optimality model apply to lysis in T7? These questions motivate our study.

Bacteriophage T7 was deleted for its lysozyme gene (*3.5*), which resulted in a profound delay in lysis time and a large reduction in fitness. A partial deletion

inactivating only lysozyme's muralytic activity yielded similar results. The debilitated phages were then adapted to higher fitness and analyzed to assess the nature of recovery of the lysis-proficient phenotype. Lysis and fitness recovery was extensive and was caused primarily by mutations in gene *16*, which is not thought to effect lysis in wild-type T7. Intermediate lysis times appear to have higher fitness than extreme times, qualitatively suggesting an optimum, though the details of the system indicate that the fit between expectations and the model observed may not reflect the assumed tradeoff.

T7 lysis.

The only T7 proteins known to function in lysis are the products of genes *3.5* (lysozyme) and *17.5* (holin). T7 lysozyme has two major functions in the phage life cycle, only one of which concerns cell lysis. T7 lysozyme also regulates late gene expression and DNA packaging by binding T7 RNA polymerase (RNAP). When lysozyme binds to T7 RNAP, it increases the rate of abortive transcription initiation, especially at class II promoters (Villemain and Sousa 1998). This leads to preferential productive transcription from class III promoters and thus to increased expression of structural genes whose products are required in large quantities late in the T7 life cycle (McAllister and Wu 1978). In turn, T7 RNAP inhibits the amidase activity of lysozyme (Cheng et al. 1994). Finally, when bound to lysozyme T7 RNAP pauses more efficiently at the CJ terminator, which plays an important role in packaging (Lyakhov et al. 1997). For this reason, production of viable phage is substantially reduced in infections with a T7 lysozyme mutant that cannot bind T7 RNAP, even if that mutant lysozyme has normal lysis activity (Zhang and Studier 2004).

T7 lysozyme (gene *3.5*) is also an amidase, which breaks up the peptidoglycan wall (Inouye et al. 1973). T7 phages lacking lysozyme generate many viable particles trapped within sedimenting material (Silberstein and Inouye 1975), although they do lyse slowly. General molecular models of cell lysis by bacteriophages suggest that lysozyme builds up inside the cell, but is blocked from access to the cell wall by the inner membrane. At some point, the phage-encoded holin, (gp17.5 in T7) triggers the permeabilization of the membrane, which exposes the peptidoglycan wall to lysozyme. The result is rapid lysis of the cell (Wang et al. 2000; Young 1992). This model is supported in T7 by the fact that cells bearing a plasmid encoding T7 lysozyme lyse when mild detergents or freeze-thawing are used to disrupt membranes (Moffat and Studier 1987). Interestingly, *17.5* mutants show only a modest delay in lysis and produce essentially normal plaques at wild-type efficiency (unpublished observations of RHH and IJM), although they have not been extensively studied.

Another potential candidate for T7 lysis is gene *16*. The product of the essential gene *16* is an internal core protein of the virion that is ejected into the host cell at the initiation of infection (Molineux 2001). The amino acid sequence similarity of the N-terminal region of gp16 with the *E. coli* lytic transglycosylase SltY led to the suggestion that gp16 plays a role in lysis (Engel et al. 1991). Gp16 has been shown to have a muralytic activity that is important at the initiation of infection (Moak and Molineux 2000; Moak and Molineux 2004). Gp16 is thought to locally hydrolyze the cell wall, thereby assisting the translocation of the phage genome from the infecting virion into the cell. Supporting this idea, mutations at glutamate 37, the catalytic residue of the lytic transglycosylase, cause a delay in genome entry under conditions where the cell wall is

more highly cross-linked, such as low temperatures or when cells are at high density (Moak and Molineux 2000). It was concluded that the mutations had no effect on lysis time that could not be explained as a result of this delay. However, the experiments were conducted using phages that contained gene *3.5*, which may mask a small effect of gp16 on lysis, and the lytic transglycosylase domain of gene 16 presents an obvious candidate for acquisition of new lytic enzyme activity. Indeed, the muralytic activity of the T4 baseplate protein gp5 plays a similar conditionally essential role in effecting T4 genome entry under sub-optimal conditions of growth (Kanamaru et al. 2005). Furthermore, mutations in T4 gene *5* can compensate for T4 *e* (lysozyme) defects (Kao and McClain 1980a; Nakagawa et al. 1985).

METHODS

Cell and phage lines.

T7 bacteriophage is a dsDNA virus with a 40 kb genome that encodes 59 proteins (Molineux 1999). In this study, we used three strains of T7: (i) a wild-type $T7^+$ (GenBank AY264774) differing from the reference line (Dunn and Studier 1983) by a one bp insertion in the non-essential gene *0.6A* (Bull et al. 2003), (ii) T7Δ3.5, lacking all but the first six codons of the 152 amino acid lysozyme gene *3.5* (Zhang and Studier 2004) and (iii) AFK136, in which codons 130-135 of *3.5* are deleted, inactivating the lysis activity of lysozyme without affecting its binding to T7 RNAP (Zhang and Studier 2004). Nucleotide numbers used here are those of wild-type T7 as given in GenBank V01146.

Plasmids with cloned phage genes were used to complement phage defects and minimize phage evolution while growing lysates. pAR4521 (Zhang and Studier 2004) carries T7 gene *3.5* downstream of its closest natural promoter, T7 ø2.5, and was used to grow the original T7Δ3.5 strain. pTP298 expresses the λ lysis genes *R, Rz* and *Rz1* from the lacUV5 promoter (Rennell et al. 1991), complementing the lack of T7 lysozyme activity (Zhang and Studier 2004), and was used to make lysates of the original AFK136 and the evolved phage T7Δ3.5_8. See below for explanation of the subscript.

Escherichia coli BL21 was used as host for plasmids. IJ1126 [*E. coli* K-12, F^-, *recC22*, *sbcA5*, *endA*, Gal^-, *thi*, Su^+Δ*(mcrC-mrr)102:*Tn*10*] was used for transfections of T7 genomic DNA. IJ1133 [*E. coli* K-12Δ*lacX74 thi*Δ*(mcrC-mrr)10*2::Tn*10*], a strain lacking type I and other restriction loci, was used as host for all experimental evolutions and other applications (Garcia and Molineux 1996).

Passaging.

Cells from frozen stocks of IJ1133 were added to a 125 ml flask containing 10 ml LB (10 g NaCl, 10 g Bacto tryptone, and 5 g Bacto yeast extract per liter) at 37° in an orbital water bath (200 rpm) and allowed to grow for one hour to a density of 1-2 x 10^8/ml, at which point 10^4-10^7 phage were added from a variable volume less than 200 ul. The culture was then incubated for 20-60 minutes, which sometimes resulted in complete lysis of the culture, before an aliquot of the infected culture was transferred to the next passage. A sample of the completed passage was treated with chloroform and stored, preserving free phage and phage particles already formed within cells. At the beginning of each day's passages, the stock from the previous passage provided the starting phage

for transfer. A subscript denotes the passage number of a phage sample. Thus, T7Δ*3.5*$_0$ (the original stock of T7Δ*3.5*) was passaged for 43 hours across 62 flasks to yield T7Δ*3.5*$_{62}$. T7 AFK136$_0$ was passaged for 25.5 hours to yield AFK136$_{43}$. T7$^+{}_0$, which had already been passaged in this laboratory under similar conditions on IJ1133 for some time, was passaged for 20.5 additional hours to create a control for the expected fitness increase in the absence of any initial genomic defect (T7$^+{}_{61}$). Some populations were analyzed as isolates (in which it is easier to determine the phenotypic effects of particular mutations), others as lysates (in which polymorphisms can be observed). Population sizes differed between passages and phage lines, making it difficult to draw conclusions about relative evolutionary rates, but because our main interest was in the attainable fitness from short-term adaptation, passaging continued until fitness increase, as estimated by the time it took passaged phage to lyse cultures from a low multiplicity of infection (moi), began to slow. We cannot rule out the possibility that further evolution might have yielded further adaptation. The mutagen N-methyl-N'-nitro-N-nitrosoguanidine was used at a concentration of 0.5 μg/ml for a single passage (T7Δ*3.5*$_{54}$) in an attempt to promote adaptation by increasing the mutation rate.

Phenotypic assays.

We measured viral fitness in a procedure similar to that used for passaging, relying on the fact that the phage population achieves a stable age-of-infection after a few phage generations. Thereafter, phage densities in the culture follow approximately exponential growth. Fitness was determined at low moi (below 0.1) across 2-5 consecutive transfers, based on the rate of increase in total phage numbers (as measured

from titers) from the end of the first or second passage to the last passage. This estimate minimizes the effect of synchronous infection, which can otherwise yield misleading fitness measures. Each fitness (doublings/hour) is calculated as $[\log_2(N_t/N_0)]/t$, where N_x is the number of phage in the flask at time x hours, corrected for dilutions over multiple transfers.

For lysis time assays, exponentially growing cells (as above) were infected with phage at a multiplicity of ~5 to achieve synchronous infection of essentially all cells. A Klett-Summerson photoelectric colorimeter (Klett) was used to measure culture turbidity at time points across the lysis window. To obtain an average lysis time, data were fitted to a cumulative normal distribution using an empirical least-squares procedure, with suitable truncation of early readings to omit the increase in turbidity that often occurs prior to the onset of lysis. (The actual fit was to $1.0- \Phi(\mu,\sigma^2,t)$, where $\Phi(\mu,\sigma^2,t)$ is the distribution function of a normal density with mean μ, variance σ^2, and integrated from 0 to t.) From this we derived both a mean lysis time of infected cells and the slope of the lysis curve at the mean (Fig. 1.2). Each reported lysis value represents at least three independent replicate curves.

Phage release assays involved infecting cells at an moi of 5, then diluting 1:100,000 after 5 minutes to decrease further adsorption. We then titered phage from samples at various time points, before and after treatment of the sample by chloroform. This procedure serves two purposes. Initially, treatment with chloroform kills cells that have been infected by phage and spares those that have not infected cells; without treatment, both will form plaques. Thus we can compare these titers to determine the

initial number of infected cells, which is required to calculate burst size. Later, after phage production has begun, the chloroformed sample is expected to form a plaque for each phage produced inside the cell, while the untreated sample will form only one plaque per infected cell. Chloroform alone did not release appreciable phage from $AFK136_0$-infected cells (Fig. 6.2C), and so for this we used lysis by egg white lysozyme and EDTA, followed by chloroform treatment. Burst sizes for each replicate were calculated as follows: (average titer of phages without chloroform treatment after phage increase stopped) ÷ (the number of initially infected cells, calculated from time points before 9 minutes).

Sequencing and statistical tests.

Sequences were determined by dideoxy chain termination reactions using ABI Big Dye mix (version 3.0) and an ABI3100 automated machine. Sequencing templates were either PCR products or the phage genome. Sequence files generated by the ABI3100 were analyzed with DNA Star software (v4.05). We sequenced the entire genome of $T7\Delta3.5_{62}$ and $T7^{+}_{61}$, gene *16* of $AFK136_{43}$, and regions of other genomes in which mutations were expected based on their presence in $T7\Delta3.5_{62}$. Primers and PCR conditions used are available upon request. All statistical comparisons used two-tailed t-tests.

Engineered recombinations.

Genomic fragment exchanges between phages were used to associate fitness and lysis effects with particular mutations. DNA from different strains of T7 was digested

with appropriate restriction enzymes, fragments were purified, complete sets of fragments were ligated, and reaction products were transfected into competent IJ1126 cells. Selected regions of phage isolates were then sequenced from PCR products to verify the recombinant status. The phages constructed by this method were (i) AFK136$_{swap}$, which is AFK136$_0$ plus four mutations from T7Δ*3.5*$_{62}$: *16*$_{G14S}$, *16*$_{Q89H}$, *16*$_{N117K}$ and *17*$_{T118A}$ (ii) T7$^+_{swap}$, which has the same four mutations of (i) but in a T7$^+_0$ genetic background, and (iii) T7Δ*3.5*,*16*$_{Q89H}$ (from an *Mlu* I fragment swap between T7$^+_0$ and T7Δ*3.5*$_{22}$) which differs from T7Δ*3.5*$_0$ by the presence of the gene *16*$_{Q89H}$ mutation and which was sequenced to ensure a lack of gene *1* mutations (Table 1.2).

RESULTS

Adaptations to compensate for lost lysozyme functions were carried out in two phage lines: T7Δ*3.5*, in which both functions of lysozyme are lost, and AFK136, in which only lytic activity is destroyed. These two adaptations can be considered replications of each other for any similarities that evolved; any differences could be due to the different starting genotype or stochastic effects. For each line we observed fitness, lysis time, and genome sequences both before and after experimental adaptation (see Table 1.2 for strain details).

Phenotypic evolution of lysis time and fitness.

On host IJ1133 at 37°, T7$^+_0$ had a fitness of 35.6 doublings/hr, and a calculated mean/median lysis time of 14.3 min. In contrast, fitness of the phage in which the entire lysozyme gene was deleted (T7Δ*3.5*$_0$) was only 10.9 doublings/hr, while lysis was

delayed to 28.2 min and highly asynchronous (Fig. 2.2, Table 2.2). AFK136$_0$ was similarly affected by loss of just the amidase activity of lysozyme, with fitness reduced to 11.4 doublings/hr and lysis delayed to 24.6 min. There was no significant difference in either phenotype between the two lysis-deficient lines prior to adaptation, suggesting that the loss of lysozyme's regulatory function had relatively little fitness effect on lysis-deficient phages compared to the loss of lysis activity. The intracellular phage yield of T7Δ*3.5*$_0$ phages is about one third that of AFK136$_0$ or wild-type T7 (Zhang and Studier 2004), which may not lead to a large effect relative to the logarithmic scale of fitness employed.

After adaptation, both mutant lines evolved to lyse more rapidly (T7Δ*3.5*$_{62}$ 12.4 min, AFK136$_{43}$ 11.7 min, Fig. 2.2, Table 2.2). Fitness also improved, evolving to 32.4 and 35.4 doublings/hr in T7Δ*3.5*$_{62}$ and AFK136$_{43}$, respectively. While there was again no significant difference in lysis time between the evolved lines, AFK136$_{43}$ had a significantly higher fitness than T7Δ*3.5*$_{62}$ ($P<0.004$; two-tailed t-test). Although it is tempting to attribute the lower evolved fitness of T7Δ*3.5*$_{62}$ to its loss of *3.5* regulatory function, replicate evolutions of each line would be required to support such a conclusion. The similarity of both evolutions is the more interesting result. Both evolved mutants lysed faster than T7$^+_0$ ($P< 0.003$; two-tailed t-test), but slower than evolved T7$^+_{61}$ (Table 2.2). In addition to a shorter time to lysis following infection, adaptation also led to more abrupt, more synchronous, lysis. This observation suggests that lysis by the parental amidase-defective phages has a stochastic component due to the loss of a control mechanism present in wild-type T7. Whether the same control mechanism was reimposed

during adaptation or whether a new mechanism evolved cannot be determined from these data.

Fitness improvement in T7Δ*3.5*$_{62}$ could be caused by evolution compensating for the lysozyme deletion and/or by adaptation to the serial passaging conditions. The host strain used in this work was an *E. coli* K-12, rather than an *E. coli* B derivative and the temperature of propagation was 37°C rather than the more usual 30° (Studier 1969). Indeed, considerable adaptation of T7^{+} to the passaging conditions occurred, from 35.6 to 41.9 doublings/hr, while lysis time also shortened, from 14.4 min to 10.1 min (Fig. 2.2, Table 2.2).

Molecular evolution.

Comparison of sequences between T7Δ*3.5*$_{62}$ and the published sequence for T7$^{+}_{0}$ revealed eight point mutations and a single base deletion (Table 3.2). Their presence was also assayed in passages 8 and 22. We considered which of these eight mutations could compensate for the lysozyme defect. None were in genes known or thought to mediate T7 lysis. Furthermore, the mutation in gene *17* has been seen in other adaptations to similar passaging conditions and hosts (Springman pers. comm.). It is thought to increase the rate of virion adsorption to cells, and is not likely to be compensatory for a lysozyme defect.

In order to identify mutations that might be non-compensatory, T7$^{+}_{0}$ and T7Δ*3.5*$_{62}$ were allowed to recombine in IJ1133. The intersection of plaques of each phage should contain recombinants from cells that were coinfected. Phages were resuspended and passaged for 10.5 hours on IJ1133 to facilitate fixation of the fittest genotype. Recombination between T7$^{+}_{0}$ and T7Δ*3.5*$_{62}$ should create a mixture of recombinant

genotypes that contain different mutations present in T7Δ*3.5*$_{62}$; outgrowth allows those mutations advantageous in the presence of lysozyme to spread through the population (Rokyta et al. 2002). The resultant lysate (T7^{+}/Δ*3.5*$_{62}$, Table 1.2) was sequenced. It contained the wild-type allele for several of the T7Δ*3.5*$_{62}$ mutations, including gene *3.5*, which is consistent with the higher fitness of T7$^{+}_{61}$ relative to T7Δ*3.5*$_{62}$. However, the recombinants carried four mutations from T7Δ*3.5*$_{62}$, two in gene *16* and one each in genes *10B* and *17*. This result suggests that these four mutations (denoted by * in Table 3.2) are adaptive under the conditions of growth and are not necessarily compensatory for the lysozyme defect. Furthermore, three of these four mutations also arose in T7$^{+}_{61}$. However, as will be shown below, the gene *16*$_{Q89H}$ mutation conferred a larger benefit in a lysozyme-deficient background and is thus in part compensatory, and we cannot rule out a similar possibility for the other mutations.

Identifying mutations compensatory for the loss of lysozyme activity.

T7Δ*3.5*$_{62}$ and AFK136$_{43}$ each carried mutations in gene *16*, which we suspected restored rapid lysis due to their presence in the muralytic domain. Some of the mutations were polymorphic within the culture, based on sequences from a number of isolates (Fig. 3.2), but every isolate carried at least two gene *16* mutations. All gene *16* mutations that evolved in the lysis-deficient lines were located in the lytic transglycosylase domain (Engel et al. 1991) of the protein. A precedent for this result is found with T4, where mutations in gene *5*, a base-plate protein that has muralytic activity, compensate for the loss of *e* gene lysozyme activity (Kao and McClain 1980a; Nakagawa et al. 1985).

We directly evaluated the effect of gene *16* mutations and found that they indeed caused rapid lysis. First, phages from the T7Δ*3.5*$_{62}$ lysate were found in which the only known difference was the presence (genotype A, Fig. 3.2) or absence (genotype B) of *16*$_{G14S}$, although both isolates also carried the *16*$_{Q89H}$ and *16*$_{N117K}$ mutations. Genotype A lysed significantly faster than genotype B, at 11.6 minutes instead of 12.4 min (Table 2.2, P<0.04; two-tailed t-test). Second, two recombinant phages were constructed *in vitro*: *16*$_{G14S}$, *16*$_{Q89H}$, and *16*$_{N117K}$ (from T7Δ*3.5*$_{62}$) were introduced into wild-type T7 and into the amidase-defective mutant AFK136$_0$ (T7$^+_{swap}$ and AFK136$_{swap}$, Table 1.2). Both phages also carry a gene *17* mutation that is not thought to affect lysis. AFK136$_{swap}$ had a fitness of 31.9 doublings/hr (Table 2.2), much higher than AFK136$_0$ (11.4 doublings/hr; P<0.0001; two-tailed t-test) and a dramatically reduced lysis time (12.9 min instead of 26.5, P<0.0001; two-tailed t-test, Fig. 4.2). However, both the fitness and lysis time of AFK136$_{swap}$ and T7$^+_{swap}$ were very similar and statistically indistinguishable. Thus, the presence or absence of gene *3.5* lysis activity is mostly or entirely masked by the gene *16* mutant proteins.

In view of these results, we considered whether the mutations *16*$_{G14S}$ and *16*$_{Q89H}$, which were initially identified as possibly non-compensatory for the lysis defect (Table 3.2; T7$^+$/Δ*3.5*$_{62}$) were actually compensatory. A phage was constructed with a lysozyme deletion and the gene *16*$_{Q89H}$ mutation (T7Δ*3.5*,*16*$_{Q89H}$, Table 1.2). The gene *16*$_{Q89H}$ mutation has a major influence on both lysis time and fitness in T7Δ*3.5*, increasing fitness by 10 doublings/hr and speeding lysis by 12 min (Fig. 5.2 and Table 2.2; compare T7Δ*3.5*$_0$ and T7Δ*3.5*,*16*$_{Q89H}$). On a log scale of fitness, this single mutation accounts for more than half the combined effects that accrued during adaptation of T7Δ*3.5*$_0$ to

T7Δ*3.5*$_{62}$. It also changes T7Δ*3.5*$_0$ fitness and lysis more than T7$^+_0$ changed during its adaptation to T7$^+_{61}$, during which time the gene *16*$_{Q89H}$ mutation also arose. Thus, while the *16*$_{Q89H}$ mutation is beneficial to a wild-type phage, it is much more beneficial in the absence of lysozyme activity. The *16*$_{Q89H}$ mutation is therefore compensatory for the loss of lysozyme activity. *16*$_{G14S}$ and the other mutations found in T7$^+$/Δ*3.5*$_{62}$ may also be compensatory, but this has not been determined.

The fitness of evolved AFK136$_{43}$ is approximately the same as that of unevolved T7$^+_0$, but it is well below that of evolved T7$^+_{61}$. Yet the lysis time of AFK136$_{43}$ is closer to the lysis time of T7$^+_{61}$ than T7$^+_0$. It is thus plausible the gene *16* mutations have pleiotropic effects that reduce fitness beyond the predicted effect from lysis time. Reducing the efficiency of genome entry is one possibility, though we lack direct evidence to support the idea.

Burst sizes and phage release.

The burst size of T7$^+_0$ was estimated at 533 phage/infected cell under these conditions of high moi, temperature and host, and was significantly greater than both that of AFK136$_{swap}$, with its burst size of 237 ($P<0.03$; two-tailed t-test) and that of AFK136$_0$, with its burst size of 81 ($P<0.005$; two-tailed t-test).

Although turbidity measurements are frequently used to monitor cell lysis (Abedon 1992; Zhang and Studier 2004), a drop in light scattering actually reflects a loss of refractility of cells and is not a measure of cell lysis *per se*. The decline in cell culture turbidity that underlies our lysis time values could therefore be caused by other factors. However, in T7$^+_0$, AFK136$_{swap}$, and AFK136$_0$, phage release and lysis time roughly

correspond (Table 2.2, Fig. 6.2). In AFK136$_0$, however, while phage escape from untreated cells follows generally the same pattern as turbidity loss, the "lysis" observed may be fundamentally different from that of the other two lines. At no point does chloroform treatment appreciably increase the amount of phage release, most likely because chloroform targets the membrane and in this line there is insufficient muralytic activity to then break through the cell wall (Fig. 6.2C). Artificial lysis by the addition of lysozyme plus EDTA, followed by chloroform, indicates that most phage production occurs by 15.5 minutes, at around the time T7$^+_0$ lyses. This result suggests that holin may be killing the host by permeabilizing the membrane and causing the loss of all cellular metabolism. This would halt intracellular phage production by 15 minutes after infection, even though phage release may continue. This finding is of fundamental importance to our interpretation of the fit of the data to the optimality model (see Discussion.)

Effect of the gene *1* mutation.

The mutation in gene *1* (T7 RNAP) was the first substitution detected in the T7Δ*3.5* line (Table 3.2). Lysozyme mutants unable to bind T7 RNAP (but with normal peptidoglycan hydrolytic activity) are greatly debilitated, and at least 18 gene *1* changes have been shown to compensate for this loss of function (Lyakhov et al. 1997; Zhang and Studier 1995; Zhang and Studier 2004). The selection for this class of gene *1* mutants by Zhang and Studier was not exhaustive, and while the *1*$_{T794A}$ mutation we found in T7Δ*3.5*$_{62}$ was not previously observed, it likely plays the same role. A relatively small effect of this mutation in the fitness assay employed here is evident by comparing T7Δ*3.5*$_0$ and T7Δ*3.5*$_8$, the latter carrying the gene *1* mutation, with respective fitnesses of

10.9 and 12.0 doublings/hr and lysis times of 28.2 and 25.4 min (Table 2.2, Fig. 5.2). These differences are not significant. Although restoration of DNA replication and packaging activities in the absence of a RNAP-lysozyme complex is fully restored by the gene *1* mutations, only a three-fold increase in intracellular phage results (Lyakhov et al. 1997; Zhang and Studier 1995; Zhang and Studier 2004). Within a lysis-defective context, this increase has little effect on fitness as measured here, which expresses fitness on a logarithmic scale in doublings per hour. The number of mutations that restore intracellular phage DNA metabolism and packaging to T7Δ*3.5*$_0$ may be sufficiently large that there is a high probability that one may sweep through the phage population before a possibly more restricted set of mutations compensating for the lysis defect.

We attempted to test the possibility that, in the fitness assay employed here, defective cell lysis masks the within-cell benefits caused by a gene *1* mutation. If this is the case, one would expect that the gene *1* mutation would have a greater effect on fitness in a phage with rapid lysis. The fitness of T7Δ*3.5*,*16*$_{Q89H}$ was therefore compared directly to T7Δ*3.5*$_{22}$. Both phages carry the *16*$_{Q89H}$ mutation, which shortens the time of lysis. However, T7Δ*3.5*,*16*$_{Q89H}$ lacks the *1*$_{T794A}$ mutation of T7Δ*3.5*$_{22}$ (T7Δ*3.5*,*16*$_{Q89H}$ also lacks *1.6*$_{R20H}$, but gene *1.6* is nonessential and has no known function). If rapid lysis amplifies the effect of a gene *1* mutation restoring intracellular DNA metabolism, T7Δ*3.5*$_{22}$ should have a much higher fitness than T7Δ*3.5*,*16*$_{Q89H}$. In contrast to this expectation, T7Δ*3.5*$_{22}$ has a fitness only 2.5 doublings/hr higher than T7Δ3.5:*16*$_{Q89H}$ ($P<0.03$; two-tailed t-test, Fig. 5.2). This difference was not significantly greater than that between T7Δ*3.5*$_0$ and T7Δ*3.5*$_8$ ($P<0.25$) by a 4 way two-tailed t-test (Bull et al. 2000). Gene *1* was sequenced in an isolate from the end of each T7Δ*3.5*,*16*$_{Q89H}$ fitness assay and no gene *1* mutation arose

during the assay. With the caveat that measurements of lysis are indirect and may not reflect enhanced phage release from cells, this result suggests that the loss of the regulatory activity of lysozyme on T7 RNAP has a relatively small effect in the fitness assay we employed.

DISCUSSION

This study illustrates the evolutionary origin of a new genetic basis for a phage fitness component, lysis. A phage deleted for an important lysis gene was adapted by serial passage to determine if and how it would improve fitness and lysis. Although the mean lysis time of the initial phage was approximately 3 times as long as that of wild-type and fitness was 25 doublings/hr less, most of the difference was compensated for during adaptation. The mean lysis times of the adapted deletion mutant and adapted wild-type phages were within 30% of each other and fitness differences were reduced to less than 20% on the $\log_2$ scale.

Evolutionary robustness, a concept similar to evolvability, is the ability to re-evolve a phenotype or to evolve new phenotypes. In this study, similar recoveries of lysis timing were seen in two phages with different, related, lysis defects, suggesting recurrence was not dependent on identical starting conditions (Lehman 2004). Although the adapted defective phages had slightly longer lysis times than the adapted $T7^{+}_{61}$, the differences were small relative to the magnitude of the original defects. This similarity can be explained by a combination of strong selection for faster lysis (relative to lysis-defective mutants) and by evolutionary robustness of the trait.

The majority of the fitness increase that occurred during adaptation was associated with gene *16*, an internal virion protein. The mutations that arose in gene *16* to compensate for the loss of lysozyme activity all lie within the lytic transglycosylase domain of the protein, which plays a role in genome entry by hydrolyzing peptidoglycan but which has no detectable effect on lysis in wild-type phage (Kemp et al. 2004; Moak and Molineux 2000; Molineux 2001). These mutations recovered much of the original phenotype, including release of phage from cells by chloroform treatment.

This observation is similar to that in phage T4. The T4 *e* gene product codes for the lysozyme (the endolysin) that helps lyse the cell at the end of an infection. T4 *e* mutants do not lyse, but suppressor mutations that alter the baseplate protein gp5 restore lysis (Kao and McClain 1980a). The gene *5* mutation conferring this phenotype has been shown to affect the lysozyme domain of gp5 (Takeda et al. 1998). Most dsDNA phages have been shown to contain a virion-associated muralytic activity that aids in genome penetration of the peptidoglycan wall at the beginning of infection (Moak and Molineux 2004), and most also code for an endolysin which, like T7 *3.5* and T4 *e* lysozyme, normally functions from inside the cell to catalyze cell lysis at the end of the infection. Thus, evolutionary robustness of lysis time may frequently involve the modification of a muralytic enzyme that normally acts from outside the cell to make it act inside.

Differences remain, however; T4 gp5 is responsible for the phenomenon known as lysis-from-without, where cells infected at a high multiplicity immediately lyse (Kao and McClain 1980a; Kao and McClain 1980b; Nakagawa et al. 1985). $T7^{+}_{0}$ does not exhibit lysis-from-without and preliminary data (not shown) suggests that $T7^{+}_{swap}$ also does not.

Although the *16*$_{Q89H}$ mutation was shown to have a major effect on the timing of lysis, and was the first *16* mutation to arise in T7Δ*3.5*, further adaptation led to the acquisition of additional mutations. Interestingly, polymorphism arose among phages in the final lysates of both T7Δ*3.5* and AFK136. All contained *16*$_{Q89H}$ or the related *16*$_{Q89L}$ but also harbored one or more additional mutation. Phages carrying *16*$_{G14S}$ lysed almost a minute, about 7%, faster than otherwise isogenic phages that lacked the mutation. However, both species coexisted in lysates, as did other combinations of gene *16* mutations in T7Δ*3.5*$_{62}$ and AFK136$_{43}$ derivatives. The variation may indicate the phage had not yet achieved the exact optimum balance between mediating lysis and performing other gp16-related functions, which include both morphogenesis and the initial steps of infection. The multiple functions of gp16 might constrain lysis time evolution. A lack of mutations of small effect might also constrain lysis time evolution to maintain polymorphism. In this case, phages with or without a mutation might straddle an optimum lysis time. At the end of some passages, such as those that ended in lysis, there was a high moi, and this introduces the possibility that polymorphism may have been maintained partially by frequency-dependent selection. Slight variations in passaging conditions are also a possibility.

It is of interest that phages SP6 and K1-5, distant relatives of T7, lack any amino acid sequence homologue to gp3.5 and code for only one protein with a lysozyme motif (Moak and Molineux 2004; Scholl et al. 2004). This motif is associated with an internal core protein that exhibits muralytic activity *in vitro* (Moak and Molineux 2004; Scholl et al. 2004). Because the enzyme activity in SP6 and K1-5 virions is different from that in T7 (lysozyme versus lytic transglycosylase) and because the activity is fused to

different virion proteins, it was suggested that muralytic activity was acquired after the T7 and SP6 phage groups had separated during evolution (Moak and Molineux 2004). This idea then predicts that a common ancestor lacked a virion-associated muralytic enzyme, although it may have harbored an endolysin function. Alternatively, like extant small single-stranded DNA and RNA phages (Young et al. 2000), this ancestral T7/SP6 may have been more dependent on host proteins to mediate cell lysis. It would be informative to determine the lysis capacity of a T7 mutant lacking both gp3.5 amidase and gp16 lytic transglycosylase activities, or that of an SP6 mutant lacking its lysozyme activity.

Phages such as SP6 and K1-5, which appear to have only a single muralytic enzyme on an internal core protein, suggest that the evolvability of gene *16* in compensating for the lack of lysozyme may be a recovery of past function – that a T7 ancestor lacked a lysozyme gene, and gp16 acted both at the initial stages of infection and as an endolysin. If so, experimental evolution of T7Δ*3.5* may have successfully recapitulated an ancestral state. This might help explain the ease with which both the regulatory and lytic functions of lysozyme are compensated. In addition, the lysis-affecting mutation 16_{Q89H} that arose in $T7^{+}_{61}$ suggests that environmental fluctuations alone, without major genomic perturbations, may favor a gene *16* with the evolutionary capacity to regulate lysis.

As a major phenotype of T7 amidase-defective mutants was a delay in lysis, a likely target of adaptation was the gene *17.5* holin. Lysis time in phage λ infected cells is controlled by the allelic state of the *S* holin gene (Chang et al. 1995). Mutations affecting holin were not found in the lysozyme-deficient lines but were observed in the adapted

wild-type $T7^{+}_{61}$, whose lysis time was faster than that of $T7^{+}_{0}$. Even if the *17.5* holin mutation speeds lysis in $T7^{+}_{61}$ (which we have not determined directly), it may not be advantageous to T7Δ*3.5*$_{62}$ or AFK136$_{43}$ if permeabilizing the membrane is not the rate limiting step to faster lysis.

Implications for evolution of the optimal phenotype.

If genetic details constrain evolution, an understanding of phenotype evolution cannot be obtained in ignorance of the underlying genetics. Lysis time is a phenotype conducive to optimality approaches (Wang et al. 1996), and we have shown here that lysis time re-evolves to approach that of the wild-type even when a major lysis gene is removed.

The prediction of an optimal lysis time is based on a tradeoff between generation time and fecundity/burst size (Abedon et al. 2001; Wang et al. 1996). Larger bursts are better, except that they increase generation time because they can usually only be achieved by delaying lysis. At a phenotypic level, this study provides a test of the optimality model, and the lysis time and fitness data can be interpreted as qualitatively supporting the model, as evidence from other phages does (Abedon et al. 2003), especially λ (Wang 2006). We have no *a priori* basis for predicting what the optimum lysis time should be, but an optimum is suggested from the fact that phages with intermediate (but short) lysis times ($T7^{+}_{0}$) had significantly higher fitnesses than those at shorter ($T7^{+}_{swap}$) and longer (AFK136$_{0}$) extremes (Fig. 4.2).

On the surface, therefore, these results qualitatively support the optimality model. Yet in understanding the molecular details, this conclusion becomes suspect. The reason

involves gene *17.5*, the holin. Under normal conditions, phage build up approximately linearly with time inside the cell, as demonstrated for ΦX174 and T4 (Hutchinson and Sinsheimer 1966; Josslin 1970) among others, and it is this accumulation on which the optimality expectation is based. Here, in contrast, abolition of gp3.5 lysis function did not greatly extend the period of phage production, probably because the holin did not evolve. Once holin permeabilizes the membrane, at roughly the time of $T7^{+}_{0}$ lysis, the host cell may cease to produce new proteins, halting phage production and eliminating the tradeoff that is a fundamental assumption of the optimality model.

There may also be additional violations of the model. It is possible that phage release is not complete in $AFK136_0$, because some phage are damaged or inextricably entangled in the cell wall, which might explain the smaller burst size of $AFK136_0$ relative to $T7^{+}_{0}$. Also, the lines were chosen because they are thought to differ chiefly in lysis mutations. However, a pleiotropic effect of the gene *16* mutations on the rate or efficiency of genome entry or phage morphogenesis cannot be ruled out.

These possible violations demonstrate the difficulties of testing optimality models with purely phenotypic approaches. Researchers often argue that expectations of optimality models are not met due to genetic constraints, but genetic constraints might also lead to results that apparently support optimality models when, in fact, the assumptions of the model are violated. The genetic details of a system must be taken into account and used to inform further work, even when empirical data qualitatively support a model. This may be particularly relevant when the parameters of a tradeoff are manipulated experimentally, under artificial conditions (environmental or genomic) to which organisms have had no time to adapt.

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Chapter 3. Alternative pathways of evolvability and genetic redundancy of lysis in T7 bacteriophage.

ABSTRACT

By knocking out genes and constraining gene networks, we can test the functional and evolutionary redundancy of genomes. If gene networks are evolutionarily redundant phenotypes may be able to re-evolve readily, and increasing constraint will have relatively little effect. One tractable phenotype is bacteriophage lysis timing, an important life history trait that controls when the virus breaks open its host cell in order to infect new bacteria. At least three genes are strongly suspected to play a role in T7 bacteriophage lysis. Gene *3.5* (lysozyme) encodes a lysin, gene *16* a secondary lysin that evolves to lyse cells effectively when *3.5* is deleted, and *17.5* produces a holin. T7 was deleted for various combinations of these genes in order to determine the initial effects as well as the mechanisms by which lysis time re-evolved. *17.5* had little effect on lysis, suggesting the presence of another holin. *3.5* and *16* were the only effective phage lysins prior to evolution. However, when a *3.5*-defective phage was allowed to evolve with *16* evolution constrained, an alternative pathway of evolvability recovered lysin activity. This study suggests that two genes (*6.3* and *19.5*) have the potential to play roles in lysis even if these roles are redundant or absent in wild-type T7.

INTRODUCTION

Very few phenotypes are controlled by only one gene. Instead, traits are formed by the interaction of a number of genes, a gene network. These networks may either involve a single gene for each function or multiple genes with overlapping functions. In either case, other genes not initially involved in the trait may have latent activities that can emerge in certain conditions, adding further complexity.

It is rarely clear how these networks come into existence and expand, contract, or diverge. One approach to this question is to remove nodes from the gene network and to observe functional redundancy. Allowing these partially dismantled networks to evolve can then also help us understand the *evolutionary* redundancy of traits. This may reveal how networks gain and lose complexity, while also revealing cryptic nodes.

Here, we use bacteriophage lysis timing as a model system. It is an important life history trait that can evolve readily and is easily assayed. Most phages, viruses that attack bacteria, need to destroy their host's cell wall in order to escape. The two-component model of lysis is general to all large dsDNA viruses thus far studied (Wang et al. 2000), although the specifics may vary somewhat (Xu et al. 2005; Xu et al. 2004). It involves two classes of proteins, holins and lysins (Young 1992). A lysin, with muralytic activity that can degrade the host's cell wall, builds up inside the cell. It is prevented from accessing the cell wall by the host's inner membrane. At the same time, a transmembrane protein known as a holin gathers in the inner membrane until it permeabilizes the membrane, allowing the lysin to pass through and degrade the cell wall. Although the molecular mechanisms of holin activity are not well understood and are likely to differ

between holins, they achieve a sudden rather than gradual release of lysin through the inner membrane (Grundling et al. 2001). This action allows the cell to continue phage production until lysis without obvious decay, whereas a gradual release of lysin would cause the cell's productivity to wane well before lysis (Josslin 1970; Wang 2006). The unabated accumulation of progeny has an obvious fitness benefit, and is general to all phages in which it has been explored, which span the range from small RNA phage φX174 to large lytic DNA phages T4 and λ (Hutchinson and Sinsheimer 1966; Josslin 1970; Wang 2006).

However, there is evidence suggesting that T7 lysis is somewhat more complicated and functionally redundant than the general model suggests (Heineman et al. 2005). Perhaps most notably, T7 failed to evolve according to an optimality model, probably due to a violation of the assumption of linear accumulation (Heineman and Bull 2007). This suggests that genetic details have a vital effect on T7 lysis, and that further dissection of the molecular mechanisms is required to understand phenotypic evolution. For this reason, T7 lysis provides an excellent system to look at both functional and evolutionary redundancy of gene networks by knocking out nodes.

Lysis in T7 seems to involve at least three proteins, a lysin, a holin, and an entry protein with transglycosylase (muralytic) activity. One, gp17.5, is the only known holin (Vukov et al. 2000). Another, lysozyme (gp3.5) is a lysin (Inouye et al. 1973). Phages lacking gene *3.5* lyse slowly and leave many mature phage virions trapped inside cells (Silberstein and Inouye 1975). This protein also binds to T7 RNA polymerase and alters gene expression (Zhang and Studier 2004). Finally, gp16 is an essential virion protein (Molineux 2001) with muralytic activity. The elimination of this activity by substitutions

at the catalytic residue can slow genome insertion (which requires phage proteins to enter the cell through a hole in the cell wall) but has little effect on lysis (Moak and Molineux 2000; Moak and Molineux 2004). However, when a phage lacking gene *3.5* was allowed to evolve, substitutions in gene *16* mediated recovery of lysis (Heineman et al. 2005), indicating gp16 has at least a latent role in lysis.

The purpose here is to (i) explore the effect of knockouts of different combinations of these genes, and (ii) observe how the defective phages evolve to overcome the defects, as a way of discovering new mechanistic layers of T7 lysis. This will allow us to study the evolutionary redundancy of lysis in response to network perturbations.

METHODS

Cell and phage lines.

IJ1126 [*E. coli* K-12, F-, *recC22*, *sbcA5*, *endA*, Gal-, *thi*, Su^+ Δ (*mcrC-mrr*) 102:Tn*10*] was used for transfections of T7 genomic DNA. IJ1133 [*E. coli* K-12Δ*lacX74* *thi*Δ(*mcrC-mrr*)102::Tn*10*] was the primary host used for all adaptations and assays. It is referred to throughout as the "normal" host when it bears no plasmid. A plasmid (pPK70) that carried a complete gene *16* insertion in vector pWSK129 (Wang and Kushner 1991) was used to express gp16 in order to complement phages lacking *16*. Note that a phage lacking *16* in its genome but which carries gp16 in its virion can infect cells and undergoes a normal transcription and replication cycle, but any virions produced are aberrant and incapable of infecting new cells if gene *16* is not provided by the host.

IJ1133 cells carrying pPK70 are referred to here as "+gp16" hosts. Two other plasmids were used to complement phage defects. pAR4521, which provides gp3.5 (Zhang and Studier 2004) was used for T7 lines lacking gene *3.5* (lysozyme). pTP298, which expresses phage λ lysis genes *R*, *Rz* and *Rz1* and complements lack of lysin activity by gp3.5, was used for the T7 strain AFK136, which contains a deletion of the enzymatic site in gene *3.5*.

The sequence of our wild-type T7 line ($T7^+$) is published (GenBank AY264774, Bull et al. 2003) and is the same as the original wild-type T7 (GenBank V01146, Dunn and Studier 1983) except for a 1 bp insertion following base 1896 in the nonessential gene *0.6*. The Dunn-Studier sequence is used as a reference for location of all changes in evolved lines. Evolved lines are denoted by a subscript E; for example, $T7^+$ adapted to our adaptive conditions is $T7^+_E$.

Deletions in T7 were generally created by growing a phage on a host carrying a pUC18 plasmid whose insert was engineered to consist of typically 100-200 bases immediately 5' of the region to be deleted, juxtaposed to the 100-200 bases immediately 3' of the region to be deleted. For example, the insert used to create T7Δ*17.5* was comprised of a PCR product containing T7 bases 35718-36343 juxtaposed to bases 36553-36933; the final PCR product was generated by a PCR reaction that joined PCR products of the respective 5' and 3' regions and lacked the holin gene *17.5*. Once this plasmid (pΔ17.5) was created, a T7 with a gene *17* amber mutation (*17am61*) which was able to form infectious progeny only on a suppressor strain, was plated on amber suppressor strain *E. coli* B argF40 SuII (IJ486) carrying this plasmid. Most

recombinations between the phage and plasmid that removed gene *17.5* would also remove the amber mutation in gene *17* and thus be able to plate on a non-suppressor strain. T7 was plated on B40Su II:pΔ17.5, one plaque was replated on IJ1133 to isolate phages that no longer required the amber suppressor, and several isolates were assayed by PCR to evaluate the *17.5* deletion. Other T7 deletion lines included T7Δ*3.5*, which lacks almost all of gene *3.5* (Zhang and Studier 2004), AFK136, in which codons 130-135 of *3.5* are deleted, eliminating lysin activity while preserving T7 RNA polymerase binding (Zhang and Studier 2004), and T7Δ*16*, a precise deletion of gene *16* (Moak and Molineux 2000).

Genomic fragment exchanges between phages were used to combine multiple gene deletions or to isolate the phenotypic effects of particular changes in adapted lines. DNA from different strains of T7 was digested with restriction enzymes, fragments were isolated, sets of fragments were ligated to regenerate genomes, and these genomes were transfected into competent IJ1126 cells. The phages constructed by this method were (1) T7Δ*3.5*Δ*17.5*, carrying the *3.5* and *17.5* deletions as well as all changes (except one at position 34975) from a T7 adapted to grow well on our environmental conditions ($T7^{+}_{61}$ from Heineman et al. 2005; here referred to as $T7^{+}_{E}$); (2) T7Δ*16*Δ*17.5*, carrying the gene *16* and *17.5* deletions; (3) AFKΔ*16*, carrying the partial deletion of *3.5* from AFK136 as well as the *16* deletion; and (4) various T7Δ*17.5* and AFKΔ*16* phages also carrying changes that arose during evolution. These phages are named by the genes in which they bear substitutions. For example, the phage identical to AFKΔ*16* except that it carries the changes in genes *0.7*, *1.6* and *1.8* that were present in an evolved AFKΔ*16* strain is

named AFKΔ*16*$_{+g0.7+g1.6+g1.8}$. (All AFKΔ*16* phages that carry one of the gene *1.8* changes carries the substitution rather than the insertion, see Table 1.3).

Passaging.

Conditions used to adapt phage lines were similar to those of Heineman et al. (2005). Basically, phages were propagated under conditions expected to select fast growth rate of the phage population, using serial passage across cultures of cells in exponential growth phase. Frozen stocks of cells were grown in 10 ml of LB at 37°C in a shaking water bath (200 rpm) to a final density of 1-2 x 10^8 cells/ml after 1 hr. At this time, 10^4-10^7 phages were added. After incubation for 20-60 min, 10^4-10^7 phages (infective centers) were added without treatment to the next flask or, if the passage was the final one for the day, it was treated with chloroform, killing cells and halting phage reproduction. Chloroform treated samples were used to resume passages on subsequent days. Passages were sometimes allowed to progress to lysis in order to facilitate recombination among phages and thereby decrease the effect of clonal interference, which can slow adaptation (Miralles et al. 1999).

The three phages adapted in this fashion were T7Δ*17.5* (7.5 hr), T7Δ*3.5*Δ*17.5* (52 hr), and AFKΔ*16* (59 hr). AFKΔ*16* was adapted on IJ1133 +gp16 cells, which compensated for the otherwise lethal deletion. In no case can we know for sure that phenotypic evolution was at an endpoint, but the fitness of the T7Δ*3.5*Δ*17.5* and AFKΔ*16* adaptation lines were no longer increasing rapidly by the end of adaptation (data not shown). An earlier study used selective conditions identical to those used in this study (Heineman et al. 2005) to adapt various phages. These evolved phages are useful

for comparison to the new adapted lines. The phages are T7Δ*3.5*$_{E}$, AFK136$_{E}$, and T7$^{+}_{E}$ (originally referred to as T7Δ*3.5*$_{62}$, AFK136$_{43}$, and T7$^{+}_{61}$ respectively).

Phenotypic assays.

Fitness here is measured as doublings/hr of the phage population, a measure proportional to the 'intrinsic rate of increase' (r) often used in ecology. The passage conditions used impose directional selection on r. This measure indicates how quickly the phage population expands (under the conditions used) and is directly comparable across different phages, regardless of generation time and other phage life history parameters, but the comparison is meaningful only when the different phages are assayed under the same conditions. In keeping with this understanding, fitness assays employed a protocol nearly identical to that used in passaging, except that the phage moi was maintained at lower than 0.1 at all times. Phages were transferred for a total of 100 min across 4-5 flasks, and titers were taken at 40 and 100 min. The initial 40 min allowed the phage population to begin growing approximately exponentially (Heineman et al. 2005). Fitness is calculated in doublings/hour as $[\log_2(N_t/N_0)]/t$, where N is the final number of phages at time t, taking into account dilutions over the course of the assay.

Lysis time assays were done as in Heineman et al. (2005) and involved infecting cells grown as above with phage at an moi of about 5 (phage density of ~ $5x10^8$/mL). At this phage density, almost all cells are infected quickly. Average time to lysis can be approximated by fitting the gradual decline in cell turbidity to a cumulative normal distribution using an empirical least squares method (Heineman et al. 2005). Decline in cell turbidity is not a measure of lysis per se. However, it is often used as a proxy

(Abedon 1992; Zhang and Studier 2004) and we have previously found lysis time to roughly parallel phage release even in T7 strains with defective lysis (Heineman et al. 2005). Cultures infected with lysis-defective phages frequently do not lyse completely, but suffer a relatively small initial drop followed by a long period of no change. Lysis "turbidity decline index" was calculated as the turbidity decrease of the culture by the endpoint divided by the turbidity maximum, which occurred shortly after phage addition. High values indicate relatively complete lysis.

For phage release assays, cells were again infected at an moi of 5. After 5 min, they were diluted by 10^5, which mostly halted further adsorption. At various time points, samples were removed and either plated immediately onto IJ1133 cells or treated with a lysis solution consisting of egg white lysozyme and EDTA, followed by chloroform addition, and only then plated. The former treatment results in a plaque for each free phage as well as each infected cell, while the latter forms a plaque for each free phage and each mature, intracellular virion that has been released from its cell by the lysis solution. In combination these measurements allow us to determine both phage release and phage production inside the cell.

Recombination assays of compensatory evolution.

Recombination assays allowed us to identify what changes that appeared during adaptation were not strictly compensatory for the deletion or other genomic modification. Specifically, these assays identified which changes remained advantageous when deleted genes were reacquired or when other mutations were present. The assay was performed by recombining an evolved phage (carrying the genomic modification and potentially

compensatory mutations) with a wild-type or other phage lacking the genomic modification and lacking compensatory mutations. (In one case both phages had adapted by different pathways and the recombination assay was used to determine the epistatic interactions between the changes associated with each pathway.) The recombination was achieved by cross-streaking both phages on a plate, thereby allowing co-infection and thus recombination at the intersection of the two streaks. The recombination creates many combinations of the evolved mutations. As the genomic modification is deleterious, the phage backbone lacking this modification will ascend, as will any of the evolved changes that are beneficial regardless of the genomic modification.

Sequencing and statistical tests.

All sequencing employed an automated ABI3100 and ABI BigDye mix (v.3.1). Sequencing was done either from PCR products or directly from the viral genome, and analyzed with DNAStar Lasergene Seqman II software (v.5.05). The entire genomes of T7Δ*17.5*$_{E}$, T7Δ*3.5*Δ*17.5*$_{E}$, AFKΔ*16*$_{E}$, and AFK136$_{E}$ were sequenced. (The latter had been previously adapted but not entirely sequenced). T7Δ*17.5*$_{E}$, unlike the other evolved lines, was sequenced and assayed from an isolate, rather than from a population. All endpoint recombination assay populations were sequenced over the regions that contained changes in the original, evolved phage, and similarly some intermediate populations of the AFKΔ*16* adaptation were sequenced over regions in which changes were present in the final phage. We also verified all genomic fragment exchanges by sequence or PCR band size. Primers and PCR conditions used available upon request. Unless indicated otherwise, all statistical comparisons were based on two-tailed t-tests.

RESULTS

Phages with deletions or partial deletions of genes implicated in lysis were characterized for their effects on lysis. The genes deleted included those coding for gp3.5 (lysozyme), gp16 (entry protein) and gp17.5 (holin). Some of these phages were then adapted to determine the nature of alternative evolutionary pathways to lysis recovery. Evolved lines were sequenced and characterized, and in many cases the effects of individual substitutions were determined.

Role of gene *17.5* in lysis.

The holin is considered to control lysis time, and assays using a phage λ system have indicated that gp17.5 of T7 acts as a holin (Vukov et al. 2000). Yet *17.5* is not essential to T7. Consistent with earlier evidence, the wild-type phage deleted for its holin gene (T7Δ*17.5*) had a lysis time of 16.6 min, 2.8 min later than that of $T7^+$ (13.8 min, $P<0.0002$; Fig. 1.3) under our assay conditions, a significant delay but far less than expected if *17.5* was the only holin. T7Δ*17.5* had a turbidity decline index of ~80%, indistinguishable from the decline of ~85% for $T7^+$. This suggests that T7 may have another holin or holin-like membrane-degrader. (Although it is possible that membrane degradation could result from something that is not a transmembrane protein and thus not strictly a holin, all such potential entities are referred to as holins here for simplicity.)

However, gp17.5 does seem to play some role in lysis, and might also have an effect on phage production inside the cell. Prior work with the T7 strain AFK136, carrying a partial deletion of *3.5* that eliminates its muralytic activity (Zhang and Studier 2004), shows that intracellular phage accumulation stops at 13.5-14.5 min, approximately

the time $T7^+$ lyses, even though AFK136 lysis is profoundly delayed beyond this time (Heineman et al. 2005). This finding contrasts with work in three other phages (T4, φX174, and λ) in which suppression of lysis results in linear phage accumulation well past the normal lysis time (Hutchinson and Sinsheimer 1966; Josslin 1970; Wang 2006). In all of these studies, lysis was delayed by changes in holin, which suggests that the cessation of accumulation in T7 AFK136 may have been because the defect was in a lysin rather than a holin. One possibility in AFK136 is that the holin gp17.5 kills the host at the normal lysis time by permeabilizing the membrane, thus halting phage growth.

To test this, we constructed a mutant with a lysis-defective lysozyme and a *17.5* deletion (AFKΔ*17.5*) and observed phage release over time in artificially lysed cells to determine when phage growth within the cell stopped. Both AFK136 and AFKΔ*17.5* production halted at similar times (Fig. 2.3), indicating that gp17.5 was not fundamental to this cessation. However, phages did escape cells more slowly in AFKΔ*17.5* than in AFK136 (Fig. 2.3), showing that gp17.5 affects lysis in defective-lysozyme phages.

Evolvability of lysis in *17.5* deletion mutants.

In light of the importance of gp17.5 to lysis, we wished to determine if T7 could easily recover from the 2.8 min delayed lysis caused by the deletion. Understanding the genetic basis of recovery could also be useful in identifying other holins in the genome. T7Δ*17.5* was adapted for 7.5 hrs to yield $T7\Delta17.5_E$, which lysed at 12 min, 4.6 min earlier than its immediate ancestor, T7Δ*17.5* ($P<0.00002$; Fig. 1.3). This phage in fact lyses more rapidly than $T7^+$ (though more slowly than $T7^+_E$, Heineman et al. 2005). Thus the lack of *17.5* does not prevent the evolution of new lysis times.

Four changes were observed in T7Δ*17.5*$_{E}$: a 2168 bp deletion of genes *0.3-0.7*, and single-base substitutions in *4.3*, *16*, and *19.5* (Table 1.3). Genomes were constructed with different combinations of these changes to determine their separate effects. The gene *16* change by itself had little effect on lysis ($P<0.52$). Combining the *16* and *19.5* substitutions (to make T7Δ*17.5*$_{+g16+19.5}$) increased lysis time two minutes over that of T7Δ*17.5*$_{+g16}$ (from 16.9 to 18.9 min, $P<0.0005$; Fig. 1). Gp19.5 is a nonessential protein of unknown function that may have endonuclease activity (Kim and Chung 1996). A role of the *19.5* substitution in lysis is not entirely unexpected, as gp19.5 may be involved in the formation of the M-hairpin loop. Deletion of the M-hairpin delays lysis by 50% of the normal time (at 30°C) and may affect cell membrane degradation, though the mechanism of this effect is unclear (Kim et al. 1997). However, the effect of the *19.5* change (in the presence of the *16* change) is in the opposite direction of that with all four changes. One possibility is that the *19.5* change may permit phage growth within the cell to continue past the time it would generally halt. If it does this by delaying the permeabilization of the membrane, it might also delay lysis.

The change in *4.3* is silent and a priori would seem to have little effect. This leaves the 2168 bp deletion of the early region to account for the 6.9 min earlier lysis of T7Δ*17.5*$_{E}$ relative to that of T7Δ*17.5*$_{+g16+g19.5}$ ($P<10^{-7}$, Fig. 1.3). Given that the *19.5* change (combined with the *16* change) slowed lysis, there may be an epistatic interaction among these changes, perhaps between the *19.5* substitution and the deletion. However, no prior work can justify an interaction between *19.5* and the deleted early genes. The deletion minimally destroys *0.3*, *0.4*, *0.5*, *0.6A* and *0.6B* and removes at least the protein kinase function of *0.7*, which has important regulatory functions. However, this early

deletion is relatively similar to those seen in a number of other adaptations, including $T7^{+}_{E}$, so there is no a priori reason to suspect that the deletion evolved specifically in response to the deletion of *17.5*. It may instead hasten genome entry, which will make lysis earlier by shortening the life cycle of the phage (Studier 1979). Based on a recombination assay, the 2168 bp deletion was advantageous even when *17.5* was present, while the *19.5* substitution was compensatory for the *17.5* deletion.

Evolvability of lysis in T7 lacking both *3.5* and *17.5*.

Recovery of fitness and lysis time in the *3.5* deletion line was fairly complete (Heineman et al. 2005), as was that of the *17.5* deletion line after even a very short adaptation. Would a phage deleted for both genes be able to recover to approximately the same extent, or would its lysis phenotype be too damaged? A phage carrying most genetic changes from $T7^{+}_{E}$ (Heineman et al. 2005) was deleted for *3.5* and *17.5* (T7Δ*3.5*Δ*17.5*) and adapted for 52 hr to produce T7Δ*3.5*Δ*17.5*$_{E}$ (Table 1.3). Lysis time recovered to 11 min, an endpoint of lysis very similar to that of T7Δ*3.5*$_{E}$, and final fitness (37.5 db/hr) was actually higher than that of T7Δ*3.5*$_{E}$ (32.4 db/hr from Heineman et al. 2005, $P<0.002$). Comparison to the T7Δ*3.5*$_{E}$ line is more relevant because T7Δ*17.5*$_{E}$ was not adapted to a point where lysis time or fitness had stopped changing rapidly. The higher final fitness of T7Δ*3.5*Δ*17.5*$_{E}$ over that of T7Δ*3.5*$_{E}$ is plausibly the result of longer adaptation rather than an intrinsic benefit to loss of gp17.5 activity, but in any case the *17.5* deletion did not greatly reduce the evolvability of the phage. Many of the changes were similar to those found in T7Δ*3.5* (Heineman et al. 2005, Table 1.3). Thus, the

absence of *17.5* was not important in this adaptation, consistent with the ease of recovery in T7Δ*17.5*$_E$.

Role of gene *16* in lysis: holin activity.

Phages with defective lysozyme (gp3.5) evolve to compensate with substitutions in the transglycosylase (muralytic) domain of gene *16* (Heineman et al. 2005). Does gp16 affect lysis in the wild-type phage? Gene *16* is essential, so a T7 lacking *16* does not form viable particles unless complemented by the host. However, a T7 whose genome lacks *16* but whose virion carries gp16 (produced from a complementing host) will lyse the cell. T7Δ*16* lysed hosts at 15.9 min, 2.1 min later than T7$^+$ ($P<0.0001$, Fig. 1.3). Lysis was complete, however, with turbidity declining by ~85%, similar to wild-type values. This delay is consistent with a model in which gp16 does affect lysis directly in otherwise wild-type phage, contrary to observations based on missense substitutions that decrease gp16's muralytic activity (Moak and Molineux 2000). However, we must entertain the possibility that the absence of gp16 during the life cycle slows lysis indirectly, rather than through any direct role in lysis.

One possibility is that an additional and undiscovered role of gp16 is as a second holin. However, the double-deletion phage of *16* and *17.5* (T7Δ*16*Δ*17.5*) lysed at 17 min. This is only 1.1 min later than T7Δ*16* ($P<0.007$) and only 0.4 min later than T7Δ*17.5* ($P<0.25$, Fig. 1.3), although lysis was somewhat less complete for this double deletion than for either of the single deletions (~70% decline in turbidity). If the two genes were both holins, lysis would be expected to be more delayed in the double deletion mutant than in T7Δ*17.5*. If they were the *only* two proteins with holin activity, lysis should be

profoundly delayed. These data indicate that gp16 is certainly not the only alternative to gp17.5 for holin activity in T7, and they question whether gp16 has substantial holin activity at all.

Role of gene *16* in lysis: lysin activity.

Gp16 is known to be at least a latent lysin (Heineman et al. 2005). If gp3.5 and gp16 are the only phage lysins, loss of both activities should be much more challenging than loss of either separately. T7 AFK136, which has a lysin-deficient *3.5*, was deleted for gene *16* to generate AFKΔ*16*. This phage had little or no detectable lysis on normal hosts, with a turbidity decline index of only around 15%. In contrast, AFK136 turbidity declined by ~70% and T7Δ*16* declined by ~85% turbidity. This interaction shows that, while the wild-type gp16 plays some small role in lysis, it is vital to lysis of cells lacking the primary lysin. Any other lysins that might exist are only slightly effective.

Evolution of lysis in a phage (AFKΔ*16*) with primary compensatory mechanism constrained.

T7 AFK136 was previously adapted in order to study compensatory evolution in response to gp3.5 lysin deficiency (Heineman et al. 2005). Evolution led to greatly reduced lysis time (from 24.6 to 11.7 min) and increased fitness (from 11.4 to 35.4 db/hr) over a relatively short time, 24.5 hr (Fig. 3.3). How is recovery affected when the primary pathway of recovery, gene *16* evolution, is constrained? AFKΔ*16* can productively infect cells carrying a plasmid encoding gp16 (+gp16 hosts). Adaptation of AFKΔ*16* allows the phage to reproduce and evolve, but no evolution of gene *16* is possible because it is

supplied in wild-type form by the host rather than inherited as part of the phage genome. This prevents the main evolutionary pathway for lysis compensation (Heineman et al. 2005).

On the complementing host, AFKΔ*16* lysed at 17.9 min and had a fitness of 23.4 db/hr (Fig. 3.3). $T7^+$ lysis was much earlier and its fitness was much higher. Since $T7^+$ fitness was relatively unaffected by whether the host provided gp16 (34.0 db/hr with gp16 in the host and 35.6 db/hr without it), the low AFKΔ*16* fitness was not primarily the result of the +gp16 hosts being adversely affected by the expression of gp16. Following adaptation of AFKΔ*16* for 59 hr, fitness increased from 23.4 to 35.2 db/hr, and lysis time shortened from 17.9 to 11.7 min, (Fig. 3.3). Fitness change was fairly gradual across the adaptation, without the steep initial rise that is frequently observed.

For comparison, AFK136 (on normal hosts, not bearing gp16) had started with a lower fitness (11.4 db/hr, $P<0.0005$) and later lysis (24.6 min, $P<0.0003$). The initial advantage of AFKΔ*16* can be attributed to the presence of large amounts of host-produced gp16, which was most likely very important to lysis when lysozyme was defective. However, $AFK136_E$ attained similar phenotypic values as $AFK\Delta 16_E$ after adaptation for a much shorter time (Fig. 3.3, Heineman et al. 2005), suggesting that the gene *16* constraint did slow adaptation. Interestingly, $AFK\Delta 16_E$ substantially regained the ability to lyse normal hosts (from ~15% turbidity decline prior to evolution to ~65% afterwards) indicating that some other gene or combination of genes may have developed lysin activity.

Alternative genetic pathways of adaptation in AFKΔ*16*$_E$.

AFKΔ*16*$_E$ was fully sequenced, revealing a number of changes, many in nonessential genes of unknown function (Table 1.3). Intermediate populations were sequenced over sites known to change in AFKΔ*16* to determine when these changes occurred (Table 2.3). No changes were detectable after 18.7 hr of adaptation despite significant fitness increase by this time (from 23.4 to 27.9, $P<0.006$), suggesting either that early beneficial changes were later lost or that several of the final substitutions were present but individually too rare to be detected in the consensus sequence.

Data from recombination assays determined that most of the changes from AFKΔ*16*$_E$ (all except for the gene *1.8* and *17* substitutions) were compensatory for lysozyme defect rather than for having gp16 provided from a plasmid (Recombinant 1, Table 2.3). These changes therefore represented a pathway of lysis time evolvability largely independent of gp16.

It was not clear whether the changes seen in AFKΔ*16*$_E$ would still be advantageous when an adapted *16* that compensated for *3.5* defect was available. Introduction of adapted gene *16* via another recombination assay resulted in the spread of the adapted *16* and the loss of all of the changes seen in AFKΔ*16*$_E$ (Recombinant 2, Table 2.3). This suggests epistatic interaction between the two alternative pathways, and the superiority of the original one involving *16* evolution.

The effects of several substitutions were assayed individually or in groups of 2-3 in the AFKΔ*16* background to identify those of largest effect (Fig. 4.3). While epistasis might affect the importance of these substitutions in different genetic backgrounds, substitutions that alter lysis greatly are likely to be important to lysis recovery somehow.

By this criteria, there are a minimum of three genes involved in lysis time change; either *0.7* or *1.6*, *6.3*, and either *17*, *17.5*, or *19.5*. This is different from the overwhelming dominance of changes in a single gene, *16*, seen in AFK136$_E$.

Somewhat surprisingly, the addition of the *6.3* substitution (to a line also carrying other changes) shortened lysis time from 15 min to 12.3 min ($P<0.02$). Very little is known about gene *6.3*, and it is poorly conserved in related phages. This result suggests that it may be more important than it had appeared, at least in a highly lysis-defective phage. Other changes of particular interest were the changes in *17.5* (holin) and *19.5* (which was also involved in compensation for holin deletion in the T7Δ*17.5* adaptation).

None of these phages carrying subsets of the evolved substitutions lysed cultures thoroughly (all caused turbidity declines of less than 25%) on normal hosts despite the relatively complete lysis of AFKΔ*16*$_E$ (~65% turbidity decline). This suggests that substitutions in multiple genes were required to work epistatically together to permit effective lysis in the absence of functional lysozyme and gp16.

DISCUSSION

We explored the genetic redundancy of phage T7's lysis time phenotype by adapting increasingly constrained, lysis-defective phages. The phages were capable of substantial compensatory evolution and fitness recovery in all cases, though the nature of this recovery depended on the initial defect.

Complexity of T7 holin activity.

The widely accepted general model of phage lysis is fundamentally a two-component system, with a holin permeabilizing the inner membrane with very precise timing, allowing the lysin to access and degrade the cell wall (Young 1992). The model has thus far been found to apply in all dsDNA phages, although some small phages lyse their hosts with a single protein without lysin activity (Wang et al. 2000) which in many cases seems to prevent synthesis of the cell wall (Bernhardt et al. 2002). This study reveals that lysis in T7 either differs in some fundamental way from the standard model, or that it has redundant holin functions that mask each other and lead to cell death at similar times. Gene *17.5* is the single documented holin in T7. The presence of a second holin (or entity with holin-like activity) is suggested by the fact that even *17.5* deleted phages halt growth inside the cell abruptly at around the time $T7^+$ lyses, and one likely cause of cell death is a holin. Moreover, the *17.5* deletion has a relatively small effect on lysis time. Deletions of phage holins generally delay lysis greatly, as in P1 (Schmidt et al. 1996), or prevent it almost entirely as in P22 (Rennell and Poteete 1985) and λ (Reader and Siminovitch 1971). By the simple model of bacteriophage lysis, holins are solely responsible for limiting access of lysins to the cell wall (Young 1992), so if T7 is to fit this model, another holin must be involved. While it is possible that T7 carries a gene with the normally-masked ability to prevent cell wall synthesis, as has been found in some small phages, this alternative would still implicate another gene in lysis.

While the nature of this putative extra holin is not known, gene *19.5* has been found to affect lysis in other studies (Kim et al. 1997; Kim and Chung 1996). A substitution in *19.5* slows lysis in a line deleted for *17.5*. While the direction of change is

surprising, it may be due to epistatic effects or a role in preventing premature cell death. This gene also evolves in a line with a defective *3.5* but a functioning *17.5*. Gene *19.5*, and the M-hairpin loop it affects, are worthwhile targets of further study.

Evolution of tertiary lysin(s).

T7 with defective lysozyme has delayed lysis and low fitness, both of which largely recover after adaptation mediated by changes in a secondary lysin, gene *16* (Heineman et al. 2005). Gene *16* plays only a small role in lysis in wild-type T7, but is vital when lysozyme is defective. On normal hosts, lysis with both genes knocked out was far more debilitated than with either knockout alone. Indeed, lysis was almost undetectable, suggesting that not only do gp3.5 and gp16 serve overlapping functions, they might be the only effective lysins in the cell.

An earlier adaptation of a phage with defective lysozyme led to recovery mediated by compensatory changes in the muralytic domain of an entry protein (gp16). This mechanism was straightforward and may in fact represent a reversion to an ancestral state in which there was no separate lysin (Heineman et al. 2005). When this primary pathway was blocked, lysis once again recovered, showing that lysis can evolve by many molecular mechanisms. However, changes in several genes seem to be important to this secondary pathway of evolvability, and there is no clear mechanism for the recovered lysis. While lysin activity returned, multiple genes working in combination were now required for what had previously been done by either of two genes alone. The changes involved in the secondary pathway were without exception disadvantageous when an

evolved gene *16* from the primary adaptive pathway was available. This shows powerful negative epistatic interactions between the two pathways.

Many of the changes seen in the secondary pathway were difficult to predict, especially the gene *6.3* change, which had a very large effect on lysis time. The role of *6.3* has been a mystery, perhaps because its effect on lysis is so marginal in normal genomes (and laboratory conditions). By looking at alternative pathways of evolvability, we can isolate these increasingly diffuse redundancies.

It is tempting to attribute the recovery of lysis to muralytic activity of *6.3* that requires modifications in holin (gp17.5) and/or the potentially holin-affecting gp19.5 (Kim et al. 1997) to access the cell wall, although this is purely speculative, and would run counter to the general observation that any holin will allow any lysin to act on the cell wall (Wang et al. 2000). Whatever the mechanisms of adaptation, the fact remains that a phage with only very minor lysis ability re-evolved substantial muralytic activity over a fairly short period of time. The enormous redundancy seen in such a small genome suggests that many phenotypes are likely to evolve readily.

Implications for optimality evolution.

A quantitative model for optimal lysis time assumes that virions accumulate linearly inside the cell after production begins, as seen in λ, T4 and φX174 (Hutchinson and Sinsheimer 1966; Josslin 1970; Wang 2006). Thus, later lysis increases generation time, but also increases how many progeny can be released (Wang et al. 1996). This then leads to the prediction that optimal lysis time will depend on environmental factors such as host density.

However, experimental adaptations of T7 failed to match the optimality predictions in conditions expected to select slow lysis (Heineman and Bull 2007). This may be due to the presence of redundant holins, which could have the paradoxical effect of lowering evolvability by preventing the evolution of large burst sizes to accompany late lysis. Some redundancy of lysis genes certainly exists in other phages such as λ (Wang et al. 2000), but in most it does not seem to impede adaptability. Even a point substitution in holin can greatly slow lysis in phage λ (Wang 2006), which is very different from what we observe in T7 (this study, Heineman and Bull 2007).

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Chapter 4. Testing optimality with experimental evolution: lysis time in a bacteriophage.

ABSTRACT

Optimality models collapse the vagaries of genetics into simple trade-offs to calculate phenotypes expected to evolve by natural selection. Optimality approaches are commonly criticized for this neglect of genetic details, but resolution of this disagreement has been difficult. The importance of genetic details may be tested by experimental evolution of a trait for which an optimality model exists and in which genetic details can be studied. Here we evolved lysis time in bacteriophage T7, a virus of *Escherichia coli*. Lysis time is equivalent to the age of reproduction in an organism that reproduces once then dies. Delaying lysis increases number of offspring but slows generation time, and this tradeoff renders the optimum sensitive to environmental conditions: earlier lysis is favored when bacterial hosts are dense, later lysis is favored when hosts are sparse. In experimental adaptations, T7 evolved close to the optimum in conditions favoring early lysis but not in conditions favoring late lysis. One of the late-lysis adaptations exhibited no detectable phenotypic evolution despite genetic evolution; the other evolved only partly toward the expected optimum. Overall, the lysis time of the adapted phages remained closer to their starting values than predicted by the model. From the perspective of the optimality model, the experimental conditions were expected to select changes only along the postulated tradeoff, but a trait outside the trade-off evolved as well.

Evidence suggests that the model's failure ultimately stems from a violation of the trade-off, rather than a paucity of mutations.

INTRODUCTION

Understanding the ultimate causes of phenotypic variation is one of the fundamental goals of evolutionary biologists. Optimality is a convenient tool because it allows us to focus on natural selection, which is both a powerful process in phenotypic evolution and one that is frequently amenable to a priori hypotheses. If optimality models match empirical observations, especially if they do so quantitatively, it suggests that we understand what shapes phenotype evolution (Orzack and Sober 1994). Optimality models assume that traits vary freely within relationships constrained by simple trade-offs, which otherwise allows these models to neglect the genetic and molecular bases by which phenotypes are formed and evolve. While some optimality models are genetically explicit, the accommodation of detailed mechanisms can render models less general. By neglecting genetic details, optimality models can potentially predict phenotype across a wide range of ecologies, often independent of organism.

The tool of optimality has been widely used, especially for traits thought to change relatively easily, such as behavior and life history. Optimality models of sex ratio (Charnov 1982; West et al. 2000), foraging behavior (Charnov 1976), altruism (Axelrod and Hamilton 1981), age of first reproduction (Stearns and Crandall 1981), parental investment (Trivers 1972), senescence (Novoseltsev et al. 2002) and others (Krebs and Davies 1993; Krebs and Davies 1997) have played a vital role in developing our framework of evolution. The uses of optimality theory range from yielding a broad

perspective for the foundations of evolution to providing quantitative insights about the evolution of specific traits (as with sex ratio).

The simplifications used in many optimality approaches, especially at the genetic level, have been criticized as fundamental flaws that render the optimality approach useless or suspect (Gould and Lewontin 1979; Pierce and Ollasen 1987; Walters and Martell 2004). The prior lack of resolution of this controversy may be attributed largely to insufficient knowledge of the genetic architecture of phenotypes. However, the genomics era may finally give us the tools to analyze phenotype genetics and allow us to answer the critical question behind this disagreement: does understanding life history evolution require a familiarity with genetic details, or do models assuming simple generalized trade-offs successfully describe adaptation? If genomes adapt successfully and freely enough, their idiosyncrasies perhaps can be ignored, despite or even because of the complexity of their mechanisms. However, if some traits cannot change or if limitations imposed by pleiotropy and other constraints, lack of mutations, or small population size greatly influence evolution by changing the trade-off surface or preventing adaptation along it, we must take genetic details into account.

For a number of reasons, it is difficult to test the success of optimality by observing natural populations, at least because many factors not considered by the model may vary between populations and confound the comparisons. Here, we use an experimental adaptation of a phage to test a priori quantitative predictions of a simple life history optimality model.

Model system: lysis time.

Bacteriophages or "phages" are viruses that infect bacteria. Obligately lytic phages have simple life cycles somewhat like that of plants and animals that reproduce only once (Fig. 1.4). First, during what might be considered an environmental or dispersal phase, the phage has left its "maternal" host and not yet infected a new one. The length of this period depends on environmental factors such as host density, phage diffusion rates, as well as the adsorption of the phage. The next phase, eclipse, is analogous to a juvenile phase. During this period, the phage has infected a cell, but has not yet produced any progeny. It is instead co-opting the host's cellular machinery to make the cell a phage factory. At the end of eclipse, *E* minutes after infection, phage progeny begin to be produced within the cell. Progeny numbers within the cell increase monotonically over time; a linear accumulation has been observed in each of the three phages thus far assayed (Hutchinson and Sinsheimer 1966; Josslin 1970; Wang 2006), with up to as much as 1000 progeny in phage λ (Reader and Siminovitch 1971). Although these phage progeny are fully constructed and viable, they remain trapped inside the cell. Finally, at lysis, *L* minutes after infection, the phage ruptures its host's cell wall to release its progeny into the environment, and phage production stops. The time between the eclipse time and lysis time (*L* - *E*) is the post-eclipse time.

Lysis timing impacts two viral fitness components, fecundity and generation time. If a phage lyses early, generation time is short but relatively few progeny are produced from that host. If it lyses late, it produces more progeny but at the cost of increasing its generation time. Consequently, the optimal lysis time depends on external factors, one of

the most useful being the density of hosts, which sets a lower limit on generation time (Abedon et al. 2001; Abedon et al. 2003; Wang 2006; Wang et al. 1996). The optimal lysis time parallels a result from optimal foraging in a patchy environment, in which the optimal amount of time spent in a resource patch varies depending on the density of patches (Charnov 1976). Sparse hosts increase the relative value of the current host and thus increase optimal lysis time.

Although host density is predicted to have a large effect on the evolution of lysis time, it is a property of the natural phage environment that is nearly impossible to measure and is likely to vary widely (Hambly and Suttle 2005). To overcome this difficulty, we use an experimental approach, creating environments of different, constant host density to provide a range of optima. Although these conditions are artificial, it is plausible that phages might encounter them in the wild at least briefly. The conditions are also uniform enough and designed so that plasticity in lysis time should not be a factor in the evolution (Abedon 1992). Most importantly for the test of the model, we can hold essentially all environmental conditions constant except density of permissive hosts, thus avoiding selection of unwanted traits and ensuring an ideal fit to the model assumptions.

The optimality model.

The equation for the optimal lysis time is approximately

$$\hat{L} = E + 1/\hat{r} \qquad (1a)$$

(quantitative model from Bull 2006 based on the assumptions of Wang et. al. 1996). The optimal lysis time at equilibrium ($\hat{L}$) equals the duration of eclipse time (*E*) plus the inverse of the intrinsic rate of increase of the phage ($\hat{r}$) when the phage is at the optimum. The model thus predicts that the optimal post-eclipse period (*L* - *E*) changes in response to environmental conditions that alter fitness, such as host density. The advantage of this result is that it consists of just three easily measured properties of the phage: lysis time, eclipse time, and population growth rate. The first two depend on cell physiology, but not cell density per se, whereas growth rate depends on the entire suite of passage conditions.

This model assumes a linear burst size function as in Fig. 1.4, a constant density of hosts in excess of phage density (with adsorptions according to a mass action process of collisions between bacteria and phage), and strict genetic control of lysis time. From Bull (2006), the resulting equation for asymptotic phage growth in our simplified experimental environments is $r = A(be^{-Lr} - 1)$, where *b* is burst size and *A* is the product of cell density and adsorption. The optimum is found by differentiating with respect to *b*, setting $\partial r / \partial b = 0$, and treating *L* as a function of b.

When formula (1a) is parameterized with empirical data, equality indicates that optimality conditions are potentially satisfied. It is of course possible that the equality is satisfied but the phage is not at the optimum. For example, if the phage was poorly adapted to the host, one could find a host density (hence a value of *r*) that satisfied (1a), but adaptation would improve *r* and thus reveal that the phage had not been at the optimum. Worse, when the equality is not satisfied, the formula does not give the

optimum, although it can be used to indicate the direction of the deviation from the optimum. The problem is that the optimum is $E+1/\hat{r}$ but the data give an estimate of only $E+1/r$. Without knowing what $\hat{r}$ should be, it is not possible to specify the optimum. However, if the phage has reached close to its maximum r, further changes will not greatly affect the values. If the relevant parameters of the phage life history are known (including the linear accumulation function of Fig. 1.4), the optimum can be calculated directly.

Some key assumptions in this model are: (i) the phage accumulation function is fixed and linear; (ii) hosts are at constant density and in great excess of phage; (iii) infected cells lyse exactly *L* minutes after infection, without any phenotypic variance. The first assumption has been relaxed somewhat by allowing two alternative linear phage accumulation functions. This simple extension does not affect optimality conditions for our purposes here (Bull 2006). The second assumption is not realistic for natural populations but can be enforced experimentally. The third assumption can be relaxed and the model solved for normal and gamma distributions of lysis times (Appendix 1). With a normal distribution of post-eclipse times (*X*), the optimum is given by

$$\hat{L}=E+\hat{X}, \tag{1b}$$

where

$$\hat{X}=\frac{1}{\hat{r}}+r\sigma^2 \tag{1c}$$

and σ^2 is the variance in lysis time between infections (Appendix 1). Although this result is exact, the assumption of normality violates phage biology because negative values of X (which are allowed by normality) are assigned negative burst sizes in the model. So the result is biologically feasible only for $r\sigma^2 < 1$ at best (Appendix 1). If the distribution of post-eclipse times is gamma, the optimum satisfies (1b), where $\hat{X}$ is the solution to

$$1 + \hat{r}\hat{X} + 2\hat{r}\frac{\sigma^2}{\hat{X}} - 2\hat{X}\left(\hat{r} + \frac{\hat{X}}{\sigma^2}\right)\ln\left(1 + \hat{r}\frac{\sigma^2}{\hat{X}}\right) = 0. \qquad (1d)$$

Since the gamma distribution does not allow negative values, this result can be applied generally. Unfortunately, (1d) cannot be solved explicitly, but it does offer a useful limit and approximations. The limit of $\hat{X}$ as $\sigma^2 \to 0$ is $1/\hat{r}$. (1c) is the σ^2 first order approximation of $\hat{X}$, and a further approximation is possible under restrictive conditions on r and σ^2 (Appendix 1). If all else fails, (1d) is easily solved numerically.

Bacteriophage T7.

The phage used here is the obligately lytic T7, which infects many lab strains of *Escherichia coli.* Its genome is 40 kb double-stranded DNA with approximately 60 genes (Molineux 1999). The phage encodes its own DNA polymerase and RNA polymerase (both having been adopted for use in molecular biology), and its genome regulation has been the subject of a virtual infection model (Endy et al. 2000). Even for a phage, T7 is

remarkably fecund, and adapted strains can increase by a trillion-fold per hour (this paper and Heineman et al. 2005). Phages similar to T7 make up a large proportion of the global phage population, which in turn makes up a large amount of the world's biomass (Suttle 2005).

Mechanisms of lysis timing.

Lysis is better understood at a biochemical and genetic level than almost any other non-trivial life history trait. At a superficial level, the molecular basis of lysis is similar among many phages, despite the fact that lysis appears to have evolved independently many times (Young 1992; Fig. 2.4). In phages with all but the smallest genomes, lysis is effected by two molecules, an enzyme to degrade the cell wall (lysin) and a timer (holin). A lysin produced by the phage has the enzymatic activity to break down the cell wall; four different families of phage hydrolases are known that have this activity (Nelson et al. 2006). However, the cell wall lies outside the inner membrane, and lysins do not have the ability to get past the inner membrane by themselves. Thus, a second type of molecule is involved as well, the holin, a membrane protein that creates pores in the inner membrane. Interestingly, the holin does not appear to create pores gradually, but triggers membrane permeabilization suddenly (Grundling et al. 2001). This permeabilization allows the lysin to access the cell wall, where it quickly lyses the bacterial host (Young 1992). In some phages, the lysin is exported first and then activated by a holin, which preserves the status of holin as timekeeper (Xu et al. 2004).

In bacteriophage λ, lysis timing can change greatly based on single mutations in the holin, suggesting that lysis time may in many cases be capable of rapid evolution

(Ramanculov and Young 2001; Wang 2006). Those studies have also demonstrated little apparent pleiotropy between lysis time and other traits. Collectively, therefore, the molecular basis of lysis in many phages matches many assumptions in the optimality model, including the genetic malleability of lysis timing. The molecular basis of lysis in phages does not provide the logic for the *linear* accumulation of phage progeny in the cell following eclipse, so that assumption is based on phenotypic observations from 3 unrelated phages (Hutchinson and Sinsheimer 1966; Josslin 1970; Wang 2006).

Although T7 is a well-studied phage, its mechanism of lysis is only partly known (Heineman et al. 2005; Inouye et al. 1973). T7 encodes a formal lysin (gp3.5, an amidase) and at least one holin, gp17.5 (Inouye et al. 1973; Vukov et al. 2000; Wang et al. 2000; Young 1992). Deletion of the lysin gene causes a profound delay in lysis, which can be recovered by mutations in the transglycosylase domain of the entry protein gp16 (Heineman et al. 2005). Even though gp17.5 has been shown to act as a holin in a λ system (Vukov et al. 2000), deletion of gene *17.5* has surprisingly little effect on lysis time (see Chapter 3), suggesting that either the phage encodes another holin or the mechanism of lysis in T7 does not fit the general model. Our use of the optimality model in this experimental study of T7 was based on a detailed understanding of lysis mechanisms in other phages and the suggested similarity of T7 lysis to those systems. The level of informed generalization motivating this study exceeds that typically used for optimality modeling in other systems.

Expectations.

From the collective knowledge and work presented above, we expected that lysis time in T7 would be able to evolve rapidly and independently of other traits. Furthermore, from the perspective of our model, we expected that, once T7 was adapted to the passage conditions, changes in host density would select changes in only the correlated phenotypes of lysis time and burst size. At a sequence level we expected changes in lysis time to map to the holin gene, *17.5*.

METHODS

Cell and phage lines.

All nucleotide numbers presented are those of wild-type T7 (T7+, GenBank V01146, Dunn and Studier 1983). IJ1133 [*E. coli* K-12Δ*lacX74 thi*Δ*(mcrC-mrr)102*::Tn*10*], a strain lacking type I and other restriction loci, was used as the permissive host for all experimental evolutions and many other applications (Garcia and Molineux 1996). In the adaptation to mixed permissive and nonpermissive cells, the nonpermissive cells were the K-12 strain IJ1517 trxA::Kn. This latter strain served only as a sink for phage; all infections fail to release phage progeny due the absence of the essential DNA polymerase cofactor, thioredoxin (Chamberlin 1974). Even after adaptation, the phage did not form plaques when plated on cells lacking thioredoxin, so the host remained non-permissive throughout (unpublished data). IJ1126 [*E. coli* K-12, F^-, *recC22*, *sbcA5*, *endA*, Gal^-, *thi*, Su^+Δ*(mcrC-mrr)102:*Tn*10*] was used for transfections of T7 genomic DNA. The three phage lines evolved are designated $T7_{Hi}$ (adaptation to high

density), $T7_{Lo}$ (formal low density adaptation), and $T7_{pLo}$ (preliminary low density adaptation) as explained below.

Passages.

Cells from recently thawed -80°C 20% glycerol LB stocks were added to a 125 ml flask containing 10 ml LB media (10 g NaCl, 10 g Bacto tryptone, and 5 g Bacto yeast extract per liter) at 37° in an orbital water bath (200 rpm) and allowed to grow for one hour to a density of 1-2 x 10^8 viable cells/ml before phage addition. Different cell concentrations and combinations were used for propagating phage in the three protocols applied here. In the high host density protocol, phages were added to undiluted cultures of IJ1133 grown under these conditions. In the *preliminary* low host density treatment, the one-hour IJ1133 cultures were diluted before phage addition, as described below. Last, the formal low density treatment used a mix of permissive cells (IJ1133) diluted to 1-2 x 10^6/ml into nonpermissive IJ1517 at 1-2 x 10^8/ml.

At this one hour timepoint, 10^4-10^7 phages (almost always more than 10^5) were added to the flask. The culture was incubated for 20-60 minutes (usually 30), before an aliquot of the infected culture, including both 10^4-10^7 (again usually more than 10^5) free phage and infected cells, was transferred to the next flask in which cells were at the requisite density and had been incubated for one hour. The cultures were sometimes allowed to lyse in order to permit recombination and speed adaptation, but most passaging occurred at a multiplicity of infection such that few cells were infected by more than one phage. A sample of the completed passage was treated with chloroform and stored, preserving free phage and releasing phage particles already formed within

cells; aliquots transferred to new flasks were not subject to chloroform treatment except between days. Passages were typically carried for three or more hours continuously across multiple flasks before the process was halted for the day. At the beginning of each day's passages, the final stock from the previous passage provided the starting phage population for transfer.

The phage line adapted to high host density was passaged on cells at 10^8/ml for more than 35 hours and is designated $T7_{Hi}$. The *formal* low density adaptation started with $T7_{Hi}$ and used mixed cells at a combined density of ~10^8/ml (permissive cells at 10^6) for 60 hours; the end phage is designated $T7_{Lo}$. The mutagen N-methyl-N'-nitro-N-nitrosoguanidine was added at a concentration of 0.5 μg/ml in passages at 0, 12.5, 24.33, and 47 hr of this treatment to promote adaptation by increasing the mutation rate. The *preliminary* low density adaptation was initiated with a phage intermediate between $T7^+$ and $T7_{Hi}$ (an isolate from 1133E of Heineman et al. 2005) that was phenotypically indistinguishable from $T7_{Hi}$. It was propagated through a progressive series of lower and lower cell densities without any apparent phenotypic adaptation (1-2 x 10^7 cells/ml for 11 hr, 2-4 x 10^6 cells/ml for 15 hr, 1-2 x 10^6 cells/ml for 15 hr, then 5-10 x 10^4 cells/ml for 33 hr) to yield $T7_{pLo}$.

Fitness.

Viral fitness was measured in a procedure similar to that used for passaging, relying on the fact that the phage population achieves a stable age-of-infection after a few phage generations. Thereafter, phage densities in the culture follow approximately exponential growth. Fitness was determined by passage at low phage/cell ratios (not

exceeding 0.1 by the end of the transfer) across 4-5 consecutive transfers, using the rate of increase in phage titers measured from the end of the first or second passage to the end of the last passage. This estimate minimizes the effect of synchronous infection at the outset, which can otherwise yield misleading fitness measures. A fitness is given as doublings/hour, calculated as $[\log_2(N_t / N_0)] / t$, where N_t is the number of phage in the flask at time *t* hours, corrected for dilutions over multiple transfers. For determining the optimality criterion, this number was transformed to a $\log_e$ value (*r*), per minute.

Adsorption.

Adsorption assays involved adding 10^6 phages from a fresh phage lysate (no more than one day old) to cells suspended in LB in flasks as above (~10^8 cells/ml), waiting 5 minutes, and then spinning down a sample to pellet adsorbed phage. Unspun and spun suspensions at 5 min were then plated to obtain total phage and free phage densities (N_{total}, N_{free},) respectively. Adsorption α was calculated from $N_{free} = N_{total}\, e^{-5\alpha}$.

Eclipse time.

Eclipse time assays involved adding 10^7 phage to cells grown one hour to 10^8/ml; after 5.5 min, samples were taken over chloroform every 30 seconds, until approximately 1.5 min before the average lysis time of the phage. Titers were taken of the treated samples, yielding a combined estimate of free phages and intracellular phages. Eclipse time was then estimated by fitting the data numerically to a simulation that modeled adsorption, eclipse, and a linear accumulation of phage after eclipse over time. Adsorption was modeled as the product of free phage density, free cell density and the

adsorption parameter; cell density was measured at the start of the assay and assumed constant over the course of the brief assay; coinfection was ignored because the phage density was much lower than cell density; adsorption was measured separately. Parameter values for phage density, eclipse, and the slope of linear phage increase were fit by empirical least squares in which the difference log(observed phage density) – log(model phage density) was normalized by log(observed phage density) to enforce equal weighting of the squared deviations at all time points; deviations at early time points could otherwise be dominated by deviations at later time points when phage densities had increased. $T7^+$ eclipse time, for which precision was less important, was estimated as first increase of phage by more than 0.5 doublings from data gathered in a previous study (Heineman et al. 2005). That study infected cells at a multiplicity of five, and thus the data are not suited to the empirical least squares method.

Lysis time.

We used two different assays of lysis time. In the first, exponentially growing cells (as above) were infected with phage at an moi (multiplicity of infection) of ~5 to achieve rapid infection of cells. A Klett-Summerson photoelectric colorimeter (Klett) was used to measure culture turbidity at time points across the lysis window. To obtain an average lysis time, data were fit to a cumulative normal distribution using an empirical least-squares procedure (Heineman et al. 2005). This method provided mean lysis time of infected cells for the culture. Each reported lysis value is the average of at least three independent cultures.

While this method is convenient, there are two reasons that an additional method is required. First, the formula for optimal lysis time includes variance in lysis time, which cannot be accurately calculated in the presence of multiple infections or by this method. Second, multiple infections may speed lysis, due to increased expression of lysis proteins. Evidence consistent with this latter hypothesis was in fact observed when estimating the lysis time of $T7_{Lo}$ by different methods (Table 1.4).

The second method assayed lysis time in primarily singly-infected cells. 5 x 10^7 phage were added to 10mL of cells at 10^8/ml, grown 5min, then diluted 10^5-fold and 10^3-fold in separate flasks to stop adsorption. Infective centers (a mix of untreated free phage plus infected cells) were plated at various time points to determine changes in titer. Assigning upper and lower bounds to the phage densities, the phage titers observed over time can be converted into proportions, treated as cumulative probabilities. In turn, the expected cumulative probabilities were generated from a model of the adsorption and lysis process and fit by empirical least squares against the observations. The model for distribution of lysis times is described in Appendix 2. All lysis times reported here used this protocol unless stated otherwise.

Burst size.

For burst size assays, 10^6 phages were added to suspensions of exponentially growing cells in flasks (10^8 cells/ml). The mix was diluted 1000-fold after 5 minutes to curtail further adsorption. At 5.5 and 6.5 min phages were titered both before and after treatment with chloroform. Treatment with chloroform kills cells, and since 6.5 min precedes the end of eclipse, all infections fail to leave progeny; the only plaques in the

chloroform treatment derive from free phage. The initial density of infected cells can be determined by comparison of these titers. At 15.5, 16.5 and 17.5 min, chloroform-treated samples were plated to estimate phage density. Burst sizes for each replicate were calculated as: (titer of phage produced at late time points) / (the number of initially infected cells, calculated from initial time points).

Sequencing.

Sequences were determined by dideoxy chain termination reactions from PCR products using ABI Big Dye mix (version 3.1) and an ABI3100. Sequence files were analyzed with DNA Star software (v4.05). The entire genomes of $T7_{pLo}$ and $T7_{Lo}$ were sequenced, as were all sites of $T7_{Hi}$ at which its recent ancestor or descendent ($T7_{Lo}$) had mutations. Primers and PCR conditions used are available upon request.

Recombination assays to assess compensatory changes.

To determine whether the mutations evolved at low density were specifically beneficial in the low density conditions, the high host density adapted ancestor was cross-streaked with $T7_{pLo}$ or $T7_{Lo}$ to allow coinfection at the intersection. Coinfected T7 genomes recombine with high frequency. Phages from the region of intersection were resuspended and passaged briefly on IJ1133. Recombination between original and adapted lines creates a mixture of recombinant genotypes that contain different combinations of mutations; outgrowth allows those mutations advantageous in the conditions used to spread through the population (Rokyta et al. 2002). This method has been successfully used to separate mutations of positive and negative effect (Heineman et

al. 2005). Pools of recombinant phages were passaged at high host density and at low host density (0.5-1 x 10^5 cells without any nonpermissive hosts). For $T7_{pLo}$, the recombinant was passaged for 15.5 hr at high host density, 10.6 hr at low. For $T7_{Lo}$, the recombinant was passaged for 16.25 hr at high host density, 18.33 hr at low. $T7_{Lo}$ was also recombined with $T7^+$ and adapted at low host density for 18 hr. Regions that differed between the two recombined genomes were sequenced in the outgrowth populations. This sequencing was in all cases done from populations of the final lysate in order to detect polymorphism. The protocol used is demonstrated for the $T7_{Lo}$ adaptation in Figure 3.

Engineered recombinations.

Genomic fragment exchanges between phages were used to associate lysis effects with particular mutations. DNA from $T7_{Hi}$ and $T7_{Lo}$ was digested with appropriate restriction enzymes, fragments were purified, complete sets of fragments were ligated, and reaction products were transfected into competent IJ1126 cells. Selected regions of phage isolates were then sequenced from PCR products to verify the recombinant status. This method was used to generate i) $T7_{Hi}$*17.5*, which was $T7_{Hi}$ plus the mutation 24 bp upstream of *17.5* from $T7_{Lo}$, ii) $T7_{Hi}$*16*, which was $T7_{Hi}$ carrying the two gene *16* mutations from $T7_{Lo}$, and iii) $T7_{Hi}$*1, 1.2, 4.3*, which contained all mutations in genes *1*, *1.2*, and *4.3* that occurred during adaptation to low host density.

Parametric test of optimality.

The optimality criterion (1b) can be written as $\hat{L}-E-\hat{X}(r,\sigma^2)=0$, where $\hat{X}(r,\sigma^2)$ is from (1c) or (1d). When parameterized with empirical values, equality will generally

not be met. To test whether the deviations are significant, we used a parametric bootstrap approach (as these equations do not obviously lend themselves to standard ANOVA approaches, at least because of unequal error variances). We assumed normal distributions for each of the phage traits, parameterized with the observed means and variances. The sampling procedure used to obtain the data was simulated from these distributions, drawing the same numbers of observations for each trait as in the data, calculating means, and assessing how often the simulated data deviated from the optimality criterion with the opposite sign as the observed deviation. (Negative values of σ^2 were assigned a value of 0.)

RESULTS

Adaptation to high host density.

Wild-type T7 ($T7^+$) had a lysis time (measured at high moi) of 13.3 min and an eclipse time of approximately 9 min in the environments used here. In our high host density passaging conditions of 10^8 cells/ml, wild-type fitness was 35.6 db/hr ($1 / r$ = 2.4). The optimal lysis time calculated from eclipse time and fitness is 11.4 min (9 + 2.4), ignoring lysis time variance, and does not match the observed lysis time of 13.3 min (Fig. 4.4A); this discrepancy is not surprising as the phage was not adapted to these conditions. We can infer from the model that adaptation should select a shorter lysis time than 13.3 min. Furthermore, as noted above, the optimum after adaptation would be even shorter than 11.4 min because r would increase as lysis time evolved. As E is not necessarily optimized for these conditions, it may evolve as well.

$T7^+$ was adapted to high host density. The evolved phage, $T7_{Hi}$, had a fitness, lysis time, lysis variance, and eclipse time of 47.9 db/hr ($1 / r = 1.8$), 10.4 min, 0.4 min^2 and 7.1 min, respectively (Table 1.4). As expected, fitness had increased and lysis time decreased after adaptation. The eclipse time also shortened, which is likely a consequence of selection for short generation time. For example, a moderately large segment of the early region was deleted during this evolution (1479 bases, eliminating non-essential genes *0.4*, *0.5*, *0.6A* and *0.6B*, as well as parts of *0.3* and *0.7*), which might increase the rate at which the phage genome enters the cell, accelerating the entire life cycle. Fortunately, the optimum in our model includes separate terms for eclipse time (E) and growth rate (r), so the optimality criteria can be adjusted accordingly. At a lysis time of 10.4 min, $T7_{Hi}$ was close to its putative optimum of 9.1 min (Fig. 4.4B). This difference is small but (surprisingly) is statistically significant ($P<0.001$). Given the layered model assumptions underlying these estimates and test, we are hesitant to attribute biological significance to this difference, but the test does indicate high significance to it.

Adaptation to low host density.

A preliminary selection at low host density started with a phage phenotypically indistinguishable from $T7_{Hi}$, transferred it through progressively lower cell densities, ultimately down to ~10^5 cells/ml for 33 hr. The putative optimality criterion under the conditions was for a lysis time of 14.7 min, yet there was no detectable change in the lysis time of the endpoint phage ($T7_{pLo}$, data not shown). Five substitutions were found in this genome, although none proved (by a recombination test) to be specifically beneficial

at low host density. Suspecting that the small population size impeded adaptation, we modified the design to create the *formal* low density adaptation.

The formal low density selection was initiated with $T7_{Hi}$ and used a mixed population of hosts. Approximately 99% of the hosts aborted the infection so that no progeny were released. The other 1% were permissive (density of ~10^6/ml). This design allowed us to, in essence, reduce burst size and thus reduce *r* for the population while maintaining a effective phage population size larger than in the preliminary selection (albeit smaller than in the adaptation to high host density). Using the eclipse time of 7.1 min, lysis time variance of 0.4 min^2 and the fitness of 7.9 db/hr ($1/r$ = 11.0) under these conditions, optimal lysis time is putatively 18.1 min.

Evolution of lysis time and eclipse.

After 60 hr of adaptation, lysis time increased to 12.4 min, a modest and significant alteration from the 10.4 min in the initial $T7_{Hi}$ ($P<0.005$ by 1-tailed t-test, Fig. 4.4C). On the surface, this increase appears to be qualitatively consistent with the optimality model, although lysis was still 6 min too fast to satisfy the new phage's putative optimality criterion of 18.6 min. Also, about a third of this change was due to an increased eclipse time (from 7.1 to 7.8 min, $P<0.006$ by 2-tailed t-test), which is not predicted by the model. The post-eclipse period was also significantly longer in $T7_{Lo}$ (3.3 to 4.6 min, $P<0.0001$ by 4-way 1-tailed t-test, Bull et al. 2000), a result qualitatively consistent with the model.

Burst size increase.

Burst size increased significantly during adaptation to low host density, from 266 to 327 phage released per infection ($P<0.04$ by 1-tailed t-test), an increase of 23%. The post-eclipse time of $T7_{Lo}$ is 39% longer than that of $T7_{Hi}$, as though the rate of phage accumulation is actually somewhat slower overall in $T7_{Lo}$. The variances of these estimates are so large as to preclude any definitive conclusions, however.

Molecular evolution.

Seven new mutations were observed in the complete sequence of an isolate from $T7_{Lo}$ (Table 2.4). Four of these changes could not be rationalized for any effect on lysis, but two were in gene *16*, which can affect lysis in some conditions, and one was 24 bp upstream of the start of *17.5*, the holin gene.

When these mutations were evaluated in subsets, two-three of them affected lysis time, as measured by high moi lysis assays. $T7_{Hi}$*17.5*, which was $T7_{Hi}$ plus the mutation upstream of *17.5*, lysed significantly more slowly than $T7_{Hi}$ (12.3 rather than 10.5 min, $P<0.04$ by one-tailed t-test). Similarly, $T7_{Hi}$*16*, which carried the two mutations from $T7_{Lo}$ gene *16* in a $T7_{Hi}$ background, lysed more slowly than $T7_{Hi}$. (12.4 min, $P<0.0002$ by one-tailed t-test). On the other hand, $T7_{Hi}$*1, 1.2, 4.3*, which carried the four most 5' mutations in a $T7_{Hi}$ background, did not lyse more slowly than $T7_{Hi}$ (10.7 min, $P<0.14$ by one-tailed t-test).

By our recombination test of compensatory evolution conducted in a low density environment, all mutations that evolved in $T7_{Lo}$ were beneficial in a low density environment. While this result may seem redundant with the adaptation, an important

distinction between the recombination assay environment and the adaptation environment is that the assay did not use non-permissive hosts. This test thus confirms that selective pressures were similar between low host density per se and mixed host conditions, as predicted by the model, and that the lack of phenotypic evolution in the preliminary study was not due to a lack of selection.

For the recombination assay conducted at high density, the resulting phage's lysis time was indistinguishable from that of $T7_{Hi}$, as expected ($P<0.84$ by 2-tailed t-test). Of the seven new mutations from $T7_{Lo}$, none ascended to the levels seen in the low-density recombination assay: four were lost and three remained polymorphic at intermediate levels. Mutations should remain polymorphic if they are nearly neutral. Thus, no mutation evolved in the low density selection appeared to be highly beneficial at high density, indicating that all were compensatory for low density. The changes in gene *16* and upstream of *17.5* that delayed lysis were among those lost at high density and thus specifically beneficial for low density.

Recall that $T7_{Hi}$ was the ancestor of the $T7_{Lo}$ adaptation and that $T7_{Hi}$ had acquired deletions of several early genes. A possible explanation for the limited adaptation at low density was thus that the deleted genes were necessary for evolving late lysis. This explanation was ruled out with a recombination between $T7^{+}$ (carrying all the genes) and $T7_{Lo}$. Adaptation of the recombinant pool at low density led to a genome the same as $T7_{Lo}$, with the early genes deleted.

DISCUSSION

Optimality models of evolution attempt to manage complexity by reducing the organism to simple trade-off functions and reducing evolution to a deterministic process of natural selection caused by few selective forces. There are obviously factors affecting evolution besides natural selection on simple trade-offs: alternative selective forces, mutation rate, population size, migration, generation time, evolutionary history, pleiotropy, and insurmountable limits to evolution. However, it may be possible to predict phenotypes accurately without taking many genetic details into account. Our study specifically addresses this possibility using phage lysis time as a phenotype. Experimental adaptations of a phage failed to reach the expected optimum lysis time in two sets of conditions, high and low host density, although the discrepancy was large only in the latter. In all adaptations, the phage remained closer to the starting conditions than predicted.

In general, three types of explanation can be proffered for this difference between prediction and outcome: (1) the basic premise of the model is correct but the optimum has been miscalculated (and the phage really did achieve optimality); (2) the experiment was too brief for the phage population to experience the appropriate mutations; (3) the phage genome does not obey the assumed trade-off. With respect to (1), we extended the model from its earlier version to incorporate environmental variance in lysis time, but that modification does not explain the difference; there is otherwise no basis for entertaining this possibility, but we cannot exclude it. The other explanations will be addressed below.

In the high host density adaptation, $T7_{Hi}$ evolved to approximately a minute later than the predicted optimum at high host density (Fig. 4.4B). The difference was statistically significant, although the biological significance of the difference is questionable, as the suite of parameter estimation models might introduce a systematic bias. More importantly, lysis time remained several minutes faster than optimum in both low density selections. In a preliminary selection, there was little phenotypic evolution despite five substitutions. In the main (formal) low density adaptation, lysis time evolved but remained far from the putative optimum, and a trait outside the model, eclipse time, explained a third of the phenotypic change in lysis time (Fig. 4.4C).

Given the adaptation observed in the formal low density treatment and that those mutations were also shown to be beneficial in the conditions of the preliminary low density treatment, it seems likely the failure of phenotypic adaptation in the preliminary low density experiment was due to a small effective population size limiting the input of mutations. The number of phages transferred was usually on the order of 10^5, which is itself low, but further reduced by the fact that 95% of phages transferred are predicted to remain unadsorbed in 30 min at this cell density. Thus, the actual population would not have allowed many mutations to arise, and most of those would not have been exposed to selection. The fact that the mutations from $T7_{Lo}$ (evolved in the mixed host environment) spread in the recombination assay done at low density strongly suggests that mutations were the limiting factor in the preliminary low-density experiment. Of course, this impediment to adaptation in our experiment may also apply in nature at low density, if phages experience a range of host densities. In ecological theory, populations with rich

resources are often expected to be more important to adaptation than marginal populations (Kirkpatrick and Barton 1997).

Failure of $T7_{Lo}$ to match optimality.

Following the failure of the preliminary low density selection to achieve phenotypic adaptation, the formal low density selection used a design that consisted of a mix of permissive and non-permissive hosts. The effect of non-permissive hosts was merely to reduce phage population growth rate by increasing phage mortality rate, and although the specific nature of our design may be unrealistic, there are many reasons why similar causes of phage mortality could be important in natural settings (Chopin et al. 2005; De Paepe and Taddei 2006).

Significant phenotypic and genetic evolution was obtained in this low density experiment (Fig. 4.4C). The evolved phage, $T7_{Lo}$, lysed slower than its ancestor $T7_{Hi}$, but its phenotype values did not satisfy the optimality criterion. This failure could be due to insufficient time, although other evidence (below) also suggests that the trade-off is violated. The fact that the experiment transcended 280 generations and fixed seven mutations suggests that there was ample opportunity for the evolution of lysis time if that phenotype could evolve large changes easily, as it does in other phages such as λ (Wang 2006). However, evaluation of equation (A1.d) reveals that selection for the optimum is relatively weak near the optimum, far more so at low host density than at high (Fig. 5.4), so insufficient time remains a possible partial cause of the failure. However, the observed discrepancy between evolved $T7_{Lo}$ and the putative optimum appears too great to be explained by this alone.

Given that we observed the evolution of seven mutations in the formal low density adaptation, the large discrepancy between predicted and observed lysis time is especially surprising from one perspective. It seems most plausible that the typical phage environment in the wild is low host density, if only because lytic phages quickly exploit their hosts and because few natural environments are thought to sustain high absolute densities of (non-stationary phase) single bacterial species. Deviation from optimality is most expected when organisms encounter novel conditions, not when they encounter common conditions (West et al. 2000). From this perspective, therefore, a phage should be predisposed to evolve a lysis time that is appropriate for low host density, which is not what we observed.

Our predictions about the genetic changes likely to be associated with adaptation to low host density were partially met. Unexpectedly, there was no change in the coding region of the holin gene *17.5*, but the change upstream of it did delay lysis (Table 1.4). It may have some regulatory effect, although it is upstream of the ribosome-binding sequence, and there are no T7 promoters in this region. Changes in gene *16* also delayed lysis but were not necessarily expected, as the lysin activity of gene *16* has no detectable effect on lysis in $T7^+$ (Moak and Molineux 2000). However, gene *16* has effected the recovery of lysis after abolition of phage lytic activity in gene *3.5* (Heineman et al. 2005), so its contribution here is plausible.

Possibility of nonlinear phage accumulation function.

There was a pronounced failure to evolve late lysis in $T7_{Lo}$, as judged by the optimality criteria: the predicted post-eclipse period was nearly 11min, whereas the

observed was close to 4.6 min. Does this mean that T7 cannot evolve later lysis? Paradoxically, late lysis per se is never favored directly in the optimality model. Instead selection favors an increase in burst size, and a delay in lysis is an unavoidable downside of the increased burst size. The evolution of late lysis per se is in fact trivial for T7 – any change that delays the life cycle will delay lysis, for example – but there is no possible benefit unless burst size improves. The failure, therefore, must lie in the ability to increase burst size. In terms of the model, this failure would mean that progeny phage do not continue to accumulate (linearly) when T7 lysis is delayed beyond the normal time, in contrast to the evidence in three other unrelated phages.

Our genetic work with T7 may shed light on this enigma. Loss of lysin activity of the lysozyme gene (*3.5*) in T7 results in a profound delay in lysis and phage release, but no increase in burst size even when cells are artificially lysed. The simple explanation is that, with an active holin (time-keeper), cell death occurs at the usual lysis time because the holin is causing the cell to "bleed" by permeabilizing the inner membrane, but the dead cell remains intact without the lysozyme to disrupt the cell wall and lyse it. Thus the cell ceases phage production but the phages do not escape. Loss of lysozyme activity therefore delays lysis without increasing burst size. Adaptation of this lysis-defective phage might be expected to select a delay in holin timing as one avenue to increase fitness (resulting in a larger burst), but the only evolutionary outcome observed was re-evolution of lysis at the normal time (Heineman et al. 2005). We thus lack evidence that T7 progeny accumulation inside the cell is linear with time, even though a diverse set of other phages do exhibit linear accumulation. While we might have anticipated the lack of linear accumulation in T7 from our previous work, that work used T7 phages that were

profoundly and artificially debilitated and did not immediately indicate a meaningful constraint on natural evolution.

Adaptation of $T7_{Lo}$ eclipse.

Optimality models are commonly tested by comparing organisms evolved in their natural environments. While this approach has many advantages, it is also subject to complications such as selection from confounding variables, non-independence between groups, and even the neglect of taxa that evolved outside the parameter bounds of the study. An advantage of our experimental test over observational studies is that it was possible to observe evolution of multiple phenotypes in an environment designed to select just changes in lysis time and burst size. This broader perspective provides insight to the model that would otherwise be difficult to obtain.

A puzzle revealed in this fashion was the evolution of a longer eclipse time in the low density adaptation, contributing a third as much to delayed lysis as the increase in the post-eclipse time. Evolution of a shorter eclipse during adaptation of $T7^{+}$ to high density was not surprising in view of the presumed novelty of these environments to $T7^{+}$. The evolution of a longer eclipse at low density after $T7_{Hi}$ was already adapted to the cells was surprising, since the cell physiology was presumably the same in the low-density as in high-density environments. That evolution represents a failure of our optimality model to capture phenotype evolution, although in this case, the optimum is easily recalculated for any eclipse time. The evolution of eclipse time might be incorporated into a more complicated model of lysis, although such a model requires understanding the impact of different eclipse times on the (linear) phage accumulation function (Bull 2006).

Our results somewhat fly in the face of important precedents for the study of lysis time in phages (Abedon et al. 2003; Wang 2006). Those studies competed phages with different holin mutations and observed evolution of rapid lysis under expected conditions. One difference is that our study used T7, whereas those studies used T4 and λ. Both of those phages have been shown to exhibit a linear accumulation of progeny inside the cell. A second difference is those studies both started with known holin variation, ours waited for variation to arise and ascend. Third, our study tested quantitative predictions; our results were in fact in qualitative agreement with the model A more useful comparison to those precedents would thus be a long term study such as ours, but using phage λ or T4 instead of T7.

Experimental adaptations have addressed the evolution of life history traits in cellular life as well (MacLean et al. 2004; Pijpe et al. 2006; Prasad and Joshi 2003; Reznick et al. 2006; van Kleunen et al. 2002). The models tested have rarely been both a priori and quantitative, although there appears to be a trend in this direction (Carvalho et al. 1998). Protein expression levels in the bacterium *E. coli* have been experimentally shown to evolve to a quantitative optimum (Dekel and Alon 2005).

Implications for optimal virulence.

Optimal virulence models maximize parasite fitness along a trade-off between parasite transmission and host mortality (virulence). A typical prediction is that pathogens evolve to kill faster when there are many hosts available and transmission is high (Jensen et al. 2006), and this framework has been used to inform research into the evolution of human diseases (Ewald 1996). Our lysis time model is probably the simplest

quantitative one available for the evolution of virulence, if one considers rapid lysis as more virulent than slow lysis (Abedon et al. 2003). The failure of T7 to match the predictions of an optimal virulence model raises questions about the likelihood of success of generalized virulence models in other systems, especially when considering that virulence of a human pathogen is far more complicated than is lysis time of a phage. Thus, harming the host often may frequently be a side effect of pathogen reproduction, not necessarily involved in a direct trade-off with increased transmission (Sokurenko et al. 2006).

The use of vaccines that prevent infection has been suggested to favor the evolution of lower virulence to unvaccinated hosts (Gandon et al. 2003). Our formal low-density treatment used what is analogous to a perfect vaccine, from which no infecting virus escapes, and its effect was to select longer lysis time, tantamount to lower virulence.

ACKNOWLEDGEMENTS

We thank IJ Molineux, IN Wang, S Abedon, S Brown, R Springman, W Harcombe, and T Keller for helpful suggestions; A Ellington suggested the experiment to test whether genes missing in $T7_{Hi}$ were important in adaptation to low density. T Hansen suggested ways to obtain exact solutions for the effect of lysis time variation on the optimum. This study was funded by NIH GM 57756 to JJB. JJB also receives support from the Miescher Regents Professorship at the University of Texas.

Figure 1.2. Lysis curve for $T7^{+}_{0}$. The dotted line shows a representative lysis curve based on Klett values. The solid curve shows the best-fit cumulative normal curve, and the vertical line shows the estimated mean lysis time for these data.

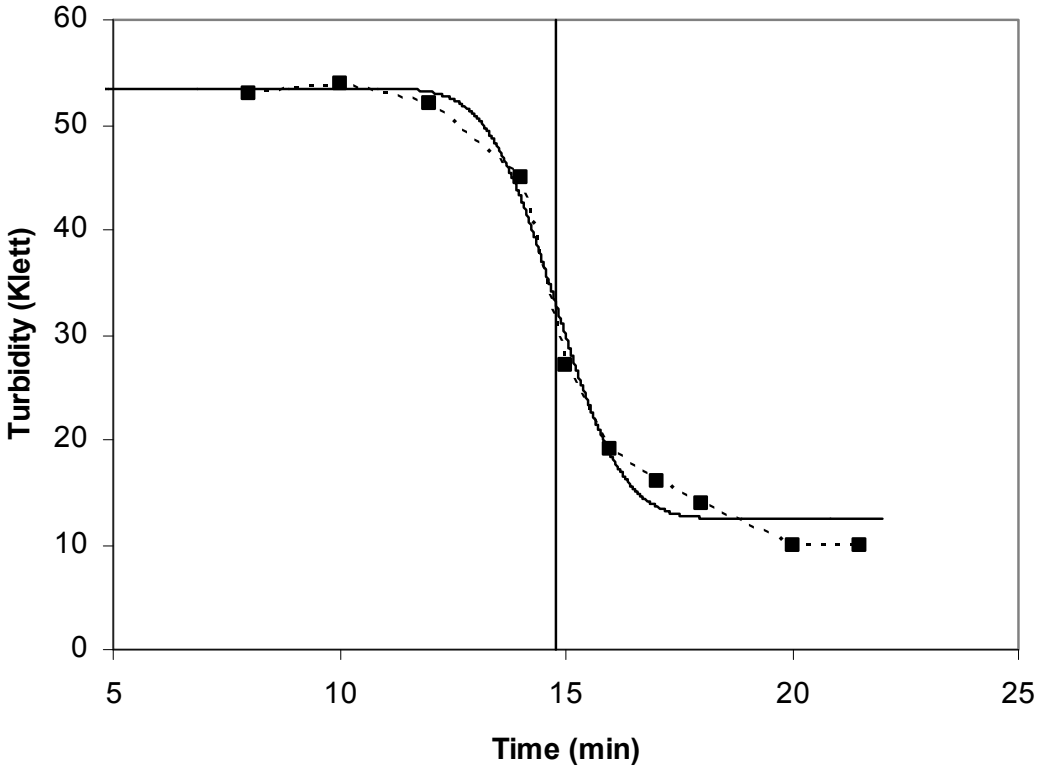


Figure 2.2. Fitness and lysis time adaptation. Solid symbols represent initial values, open symbols represent values after adaptation. T7Δ*3.5*: ○,●; AFK136: □,■; $T7^+$: ∆,▲. Each line represents the adaptation of a phage line. In all lines, lysis time decreased and fitness increased during adaptation, so the direction of adaptation is from lower right to upper left. 95% confidence bars, sometimes smaller than the point, are provided for lysis and fitness data.

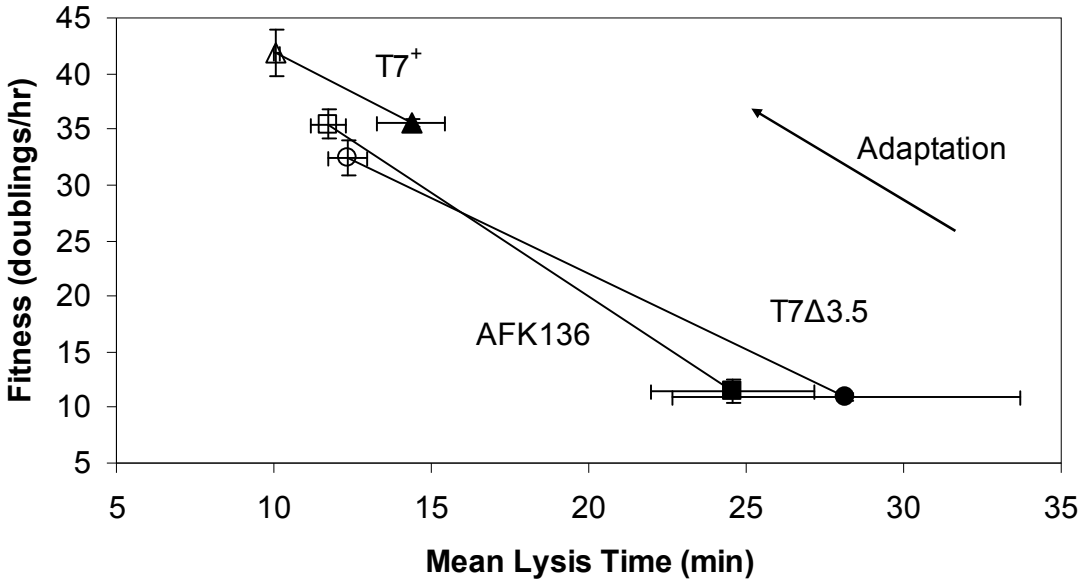


Figure 3.2. Gene *16* mutations found in individual phage genomes obtained from T7Δ*3.5*$_{62}$ and AFK136$_{43}$ lysates. The 5' end of gene *16* was sequenced using phages from several isolated plaques in order to identify polymorphisms. A filled rectangle means that the mutation was present in an individual phage genome, an empty rectangle means that it was absent. The number of purified phages found carrying a particular genotype is shown in parentheses for each genotype.

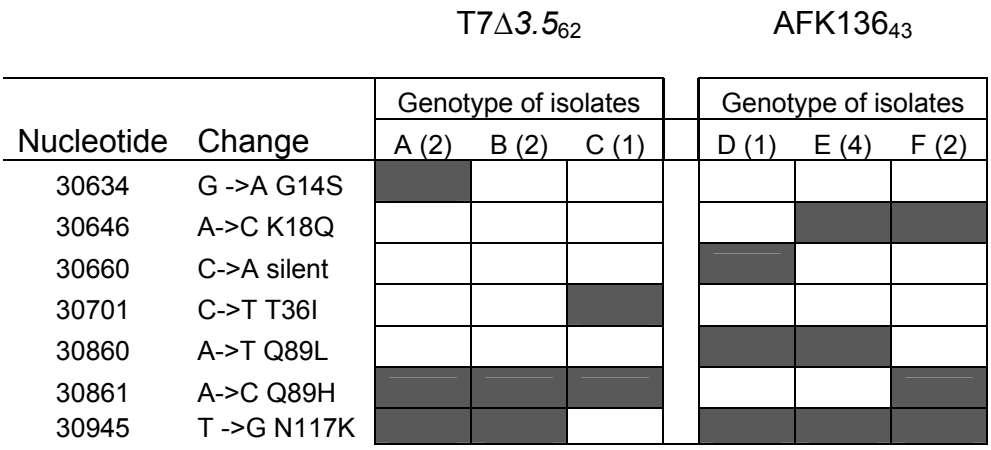


Figure 4.2. Apparent stabilizing selection for lysis time. In the absence of gene *16* mutations, lysozyme deficiency has a large effect on fitness and lysis time, which is largely rectified by gene *16* mutations. Fitness at an intermediate lysis time is higher than at more extreme times, suggesting an intermediate optimum which may actually be caused largely by violations of the optimality model.

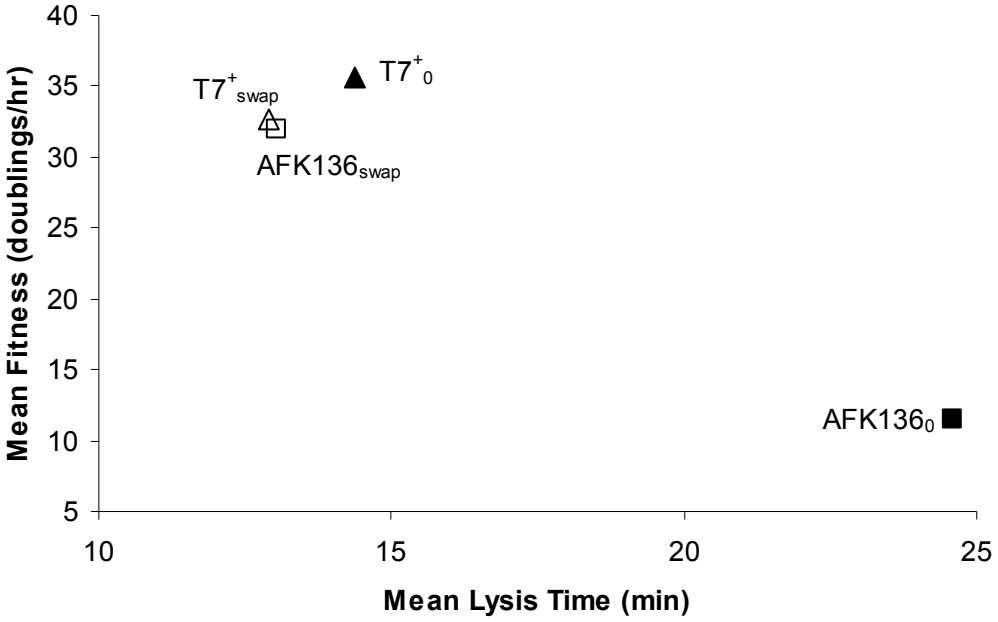


Figure 5.2. Phenotypic evolution and appearance of mutations during T7Δ*3.5* evolution. The horizontal axis indicates the times (hours of passage) during the adaptations when phage lysates were obtained, the data points correspond to passage numbers 0, 8, 22, and 62. Fitness (closed circles) and lysis time (open circles) exhibit an inverse relationship. Mutations are indicated only at the time at which they were first observed. T7Δ*3.5*,*16*$_{Q89H}$ (squares) is also shown near time 0.

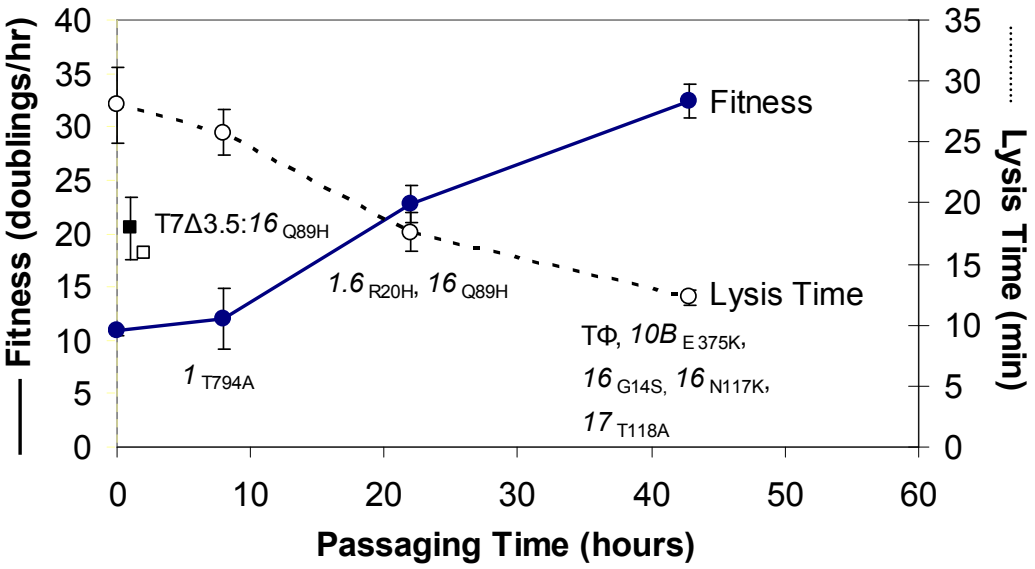


Figure 6.2. Phage release for $T7^{+}_{0}$ (A), $AFK136_{swap}$ (B) and $AFK136_{0}$ (C). Phage titer of samples treated with chloroform (squares) or untreated (circles) at various time points after infection. A different replicate of $AFK136_{0}$ (triangles) was also subjected to artificial lysis (see Materials and Methods) to ensure release of mature phage particles. This assay has a slightly lower titer than the untreated replicate shown at the beginning and end of assay, which may be caused by variance between replicates.

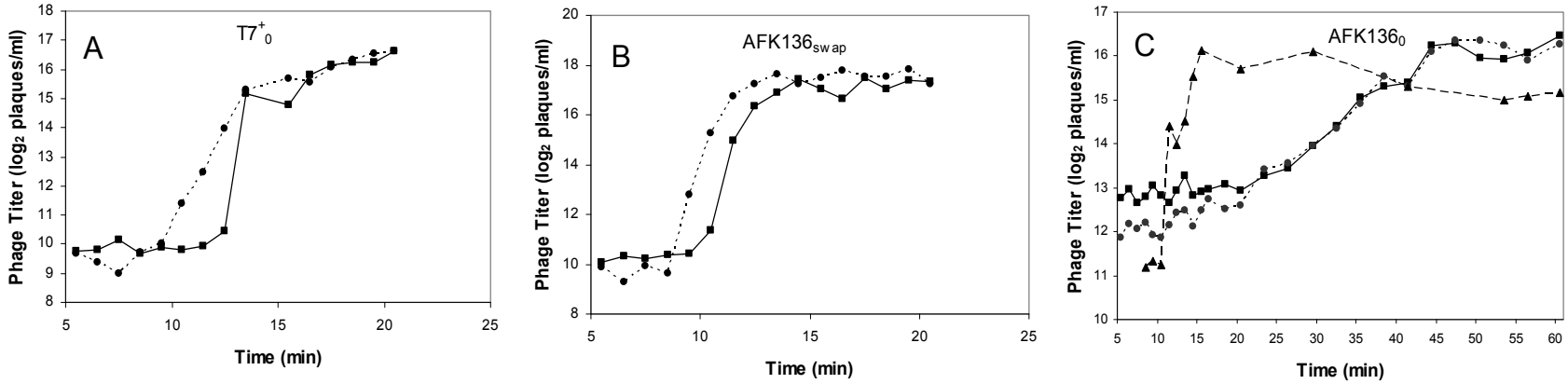


Figure 1.3. Lysis time of various phages on normal hosts. Error bars indicate one standard error. Bars are labeled with the same letter if they are statistically indistinguishable by two-tailed t-test. Gene *16* and gene *17.5* deletions both delay lysis, though deleting them both has little effect beyond merely deleting gene *17.5*. T7Δ*17.5* was adapted to yield T7Δ*17.5*$_{E}$, which has a large deletion of some early genes, a silent change in gene *4.3*, and coding changes in gene *16* and *19.5*. The gene *16* change had little effect on lysis when isolated, while adding the gene *19.5* change actually delays lysis. T7Δ*17.5*$_{E}$ lyses much faster. This suggests that the 2168 bp *0.3-0.7* deletion, the only coding difference between T7Δ*17.5*$_{+g16+g19.5}$ and T7Δ*17.5*$_{E}$, has a large effect (although this effect may be due to quicker genome entry rather than lysis per se).

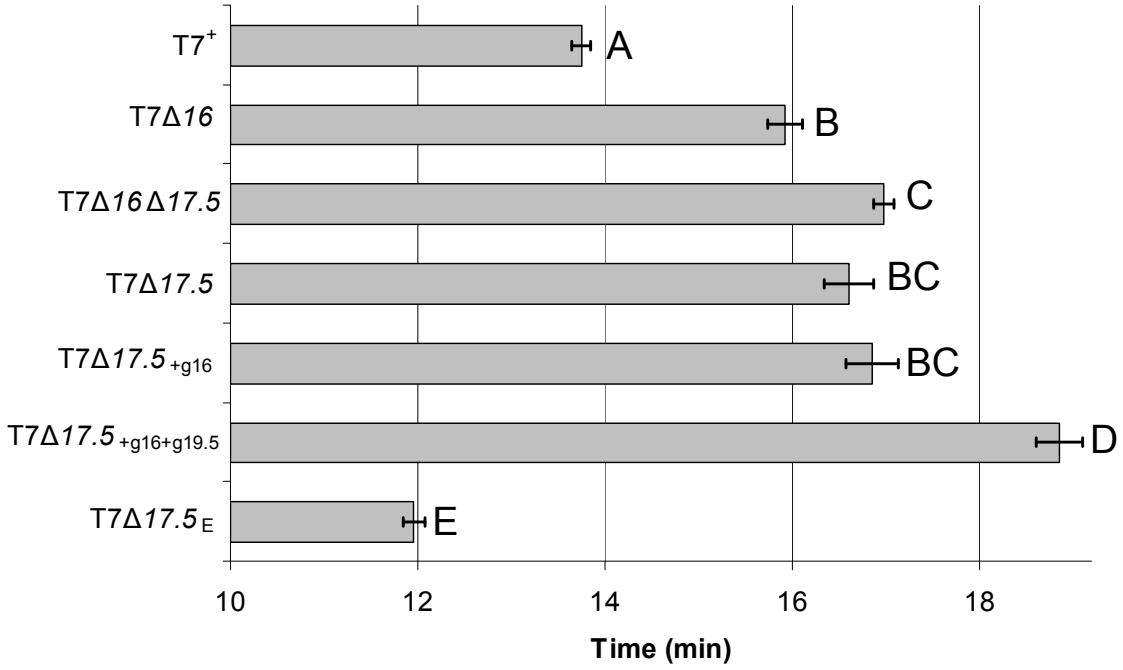


Figure 2.3. Phage release for AFK136 and AFKΔ*17.5*. Dotted lines indicate phage titer at various times after infection, a measure including both unlysed cells and free phage virions. Solid lines indicate phage titer when cells were lysed artificially to free mature phages from hosts. AFK136 is released from cells slowly, due to lysis defects (open squares). Within the cell, as seen with artificial lysis, phage production is rapid at first but halts suddenly (closed squares). AFKΔ*17.5* halts phage production within the cell at approximately the same time as AFK136 (closed circles), demonstrating that the failure to continue accumulating phage is not caused by the holin activity of gene *17.5*. The *17.5* deletion does, however, substantially decrease the rate at which phages escape the cell (open circles.)

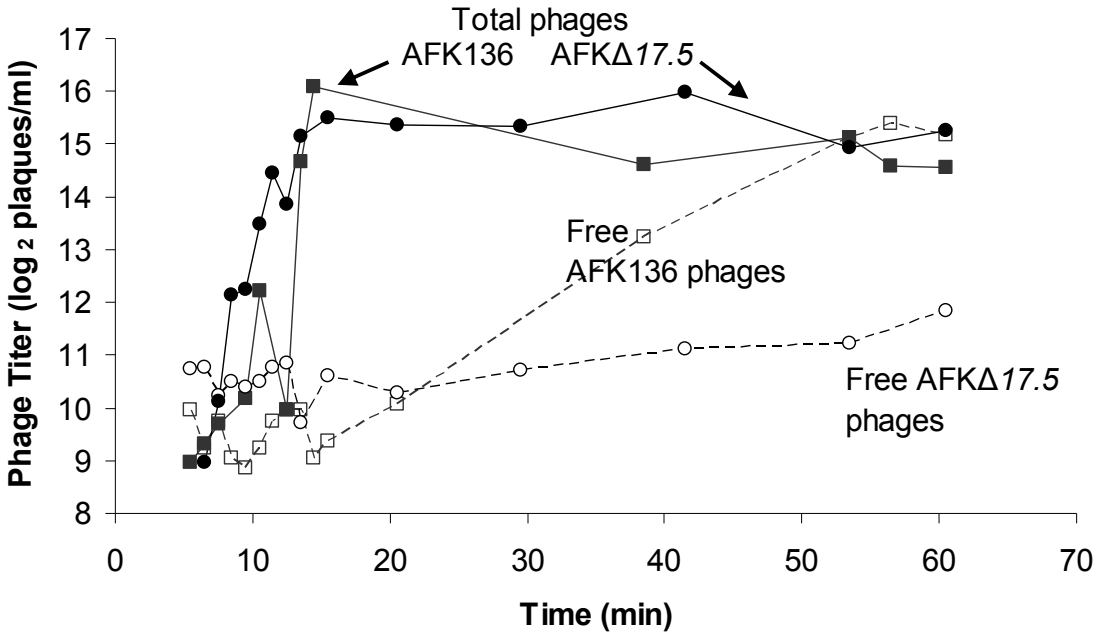


Figure 3.3. Phenotypic evolution of AFKΔ*16* (solid lines). The horizontal axis shows times when phage lysates were obtained. Fitness (closed circles) increased over adaptation while lysis time (closed squares) decreased. AFKΔ*16* evolved slowly relative to AFK136 (dotted lines, from Heineman 2005). AFK136 fitness (open circles) was actually lower than that of AFKΔ*16* before adaptation (due to the difference in hosts, as AFKΔ*16* was adapted and assayed on +gp16 cells), but it increased far more quickly. Lysis time (open squares) also evolved more quickly in AFK136. This demonstrates that prevention of gene *16* evolution was a meaningful constraint on the rate of phenotypic adaptation. Despite the significant gains in fitness by hour 18.7 in AFKΔ*16*, none of the substitutions present in the final evolved phage had yet appeared (Table 2.3), suggesting the presence of other substitutions that later disappeared due to epistatic interactions. Error bars, sometimes smaller than the point, indicate one standard error.

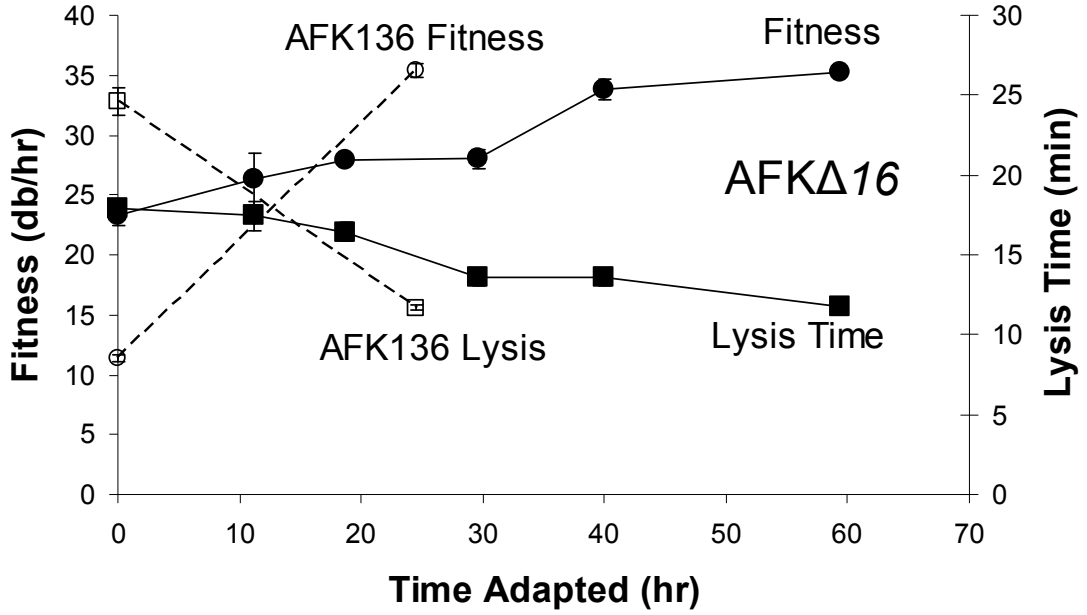


Figure 4.3. Lysis times of various phages on hosts producing gp16. (Of these phages, only AFKΔ*16*$_E$ lysed appreciably on normal hosts.) Error bars indicate one standard error. Bars are labeled with the same letter if they are statistically indistinguishable by two-tailed t-test. AFKΔ*16* lysed slowly, while AFKΔ*16*$_E$ recovered relatively rapid lysis. The gene *0.7* and *1.6* substitutions together had a large effect (2.6 min). The *0.7* substitution may have indirectly hastened lysis time by altering genome entry or might have some other regulatory effect. Adding the *1.8* change to these other substitutions had no detectable effect, which is not surprising as a recombination assay suggested it was an adaptation specifically to +gp16 hosts (unpublished data). The further addition of the *6.3* change hastened lysis by 2.7 min, suggesting this largely uncharacterized gene was very important. Finally, the *17*, *17.5*, and *19.5* changes combined delayed lysis by 3.5 min.

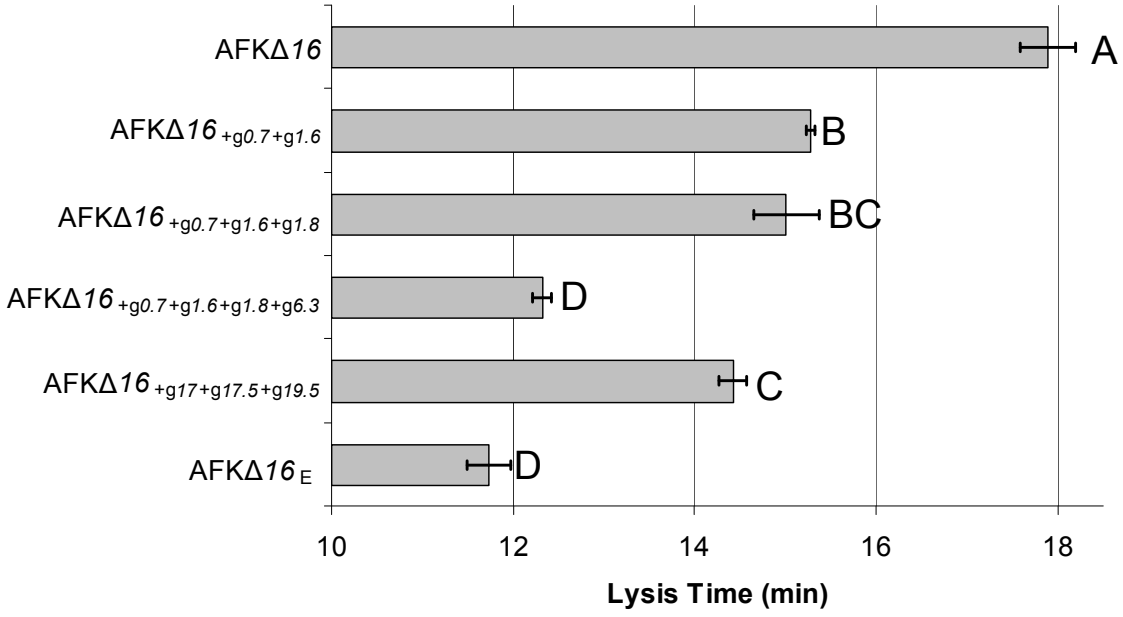


Figure 1.4. The basic life cycle of a lytic phage is analogous to that of many other organisms that reproduce only once. The environmental period, in which phage await contact with a host, is similar to dispersal. The eclipse period is the time after infection but before any viable progeny are constructed. This period, in which the host is turned into a phage factory, is much like the juvenile phase of other organisms. In the post-eclipse period, "adulthood," progeny begin to accumulate inside the cell. The phage accumulation function is linear in all known cases, meaning that, after eclipse, progeny are assembled within the cell at a constant rate. Lysis allows the viral progeny to escape the host cell at the end of an infection but halts further phage production inside the cell. Later lysis increases the amount of phage produced inside the cell but also increases generation time.

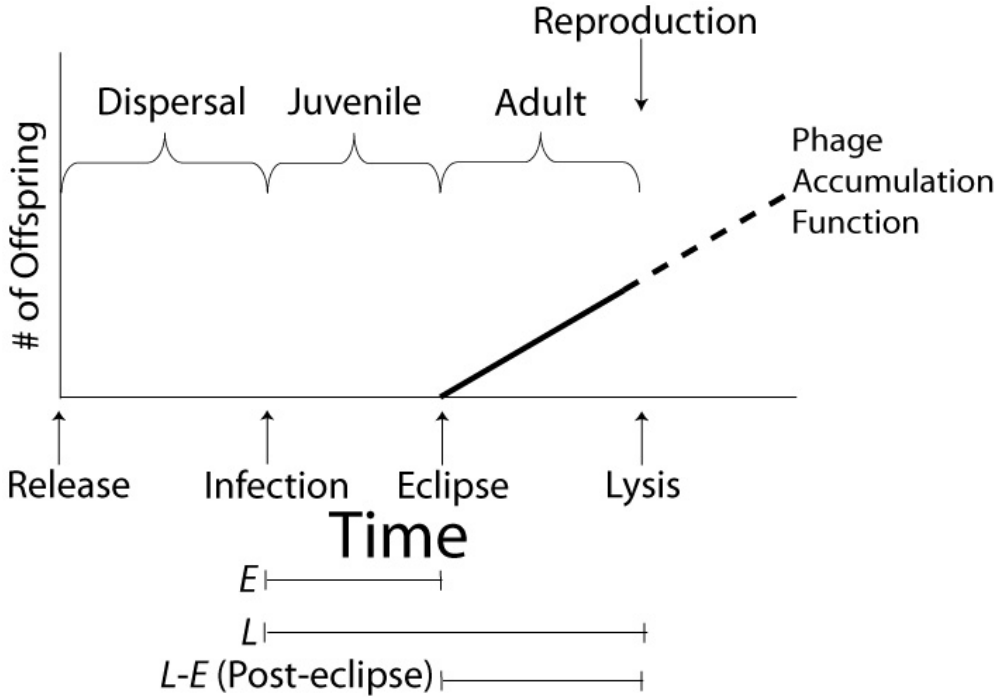


Figure 2.4. General molecular model for lysis. The lysin, which can destroy the cell wall, is at first held away from the cell wall by the inner membrane. Phage progeny continue to increase inside the cell, but are not released. Holin acts as timekeeper by at some point permeabilizing the membrane rapidly. This allows lysins to pass the inner membrane and destroy the cell wall all at once, killing the host and releasing phage. While T7 has the genetic characteristics of lysis in many other phages, its lysis mechanisms are not so well understood.

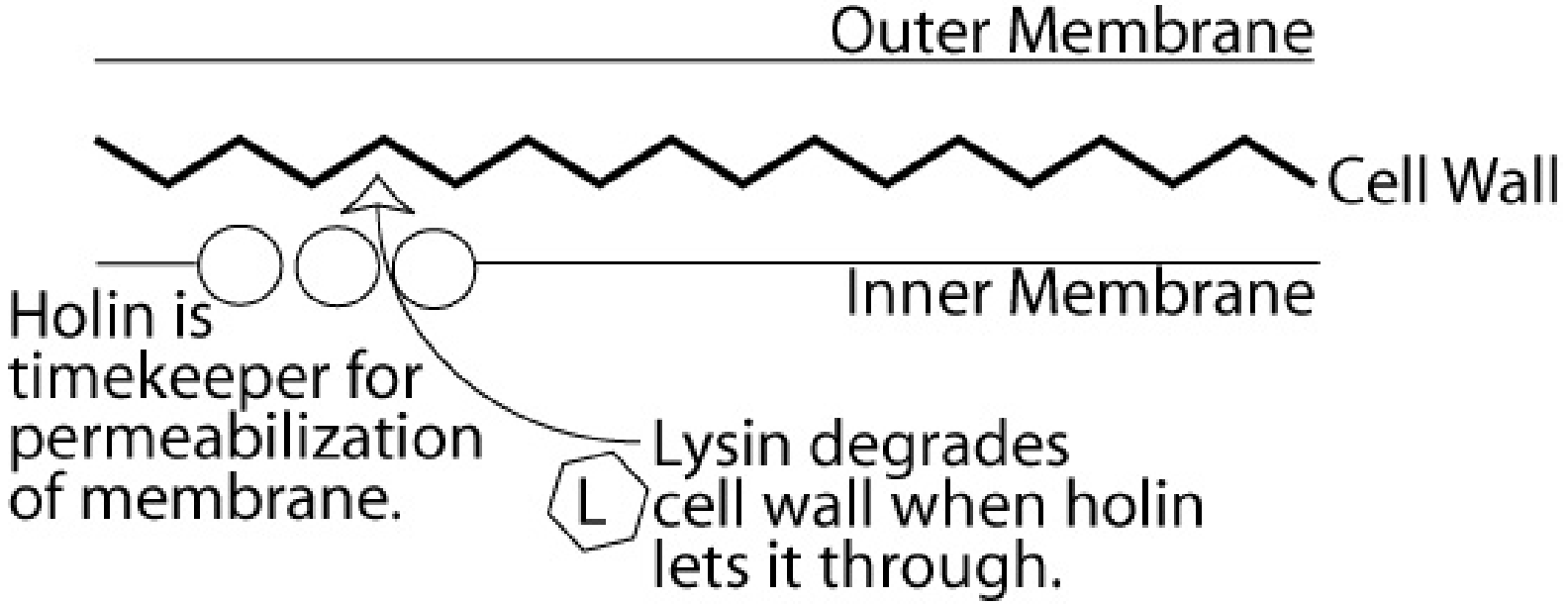


Figure 3.4. Summary of T7 adaptation and recombination design. Wild-type T7 ($T7^+$) was adapted to high host density to yield $T7_{Hi}$. $T7_{Hi}$ was adapted to mixed permissive and nonpermissive cells, expected to select for slower lysis (yielding $T7_{Lo}$, which was sequenced). A recombination assay was done between $T7_{Hi}$ and $T7_{Lo}$. The recombinant population should contain many combinations of substitutions from each genome. The recombined pool adapted to high cell density was similar to $T7_{Hi}$ in both sequence and lysis time, indicating that the substitutions that occurred during $T7_{Lo}$ adaptation were only greatly advantageous under selection for slow lysis (4 mutations were lost, 3 remained polymorphic but did not ascend to high levels). The recombinant line adapted to low cell density was indistinguishable from $T7_{Lo}$ in sequence and phenotype, suggesting that all of the mutations were highly advantageous at low host density. A recombination between $T7_{Lo}$ and $T7^+$ adapted at low density also arrived at a sequence identical to $T7_{Lo}$.

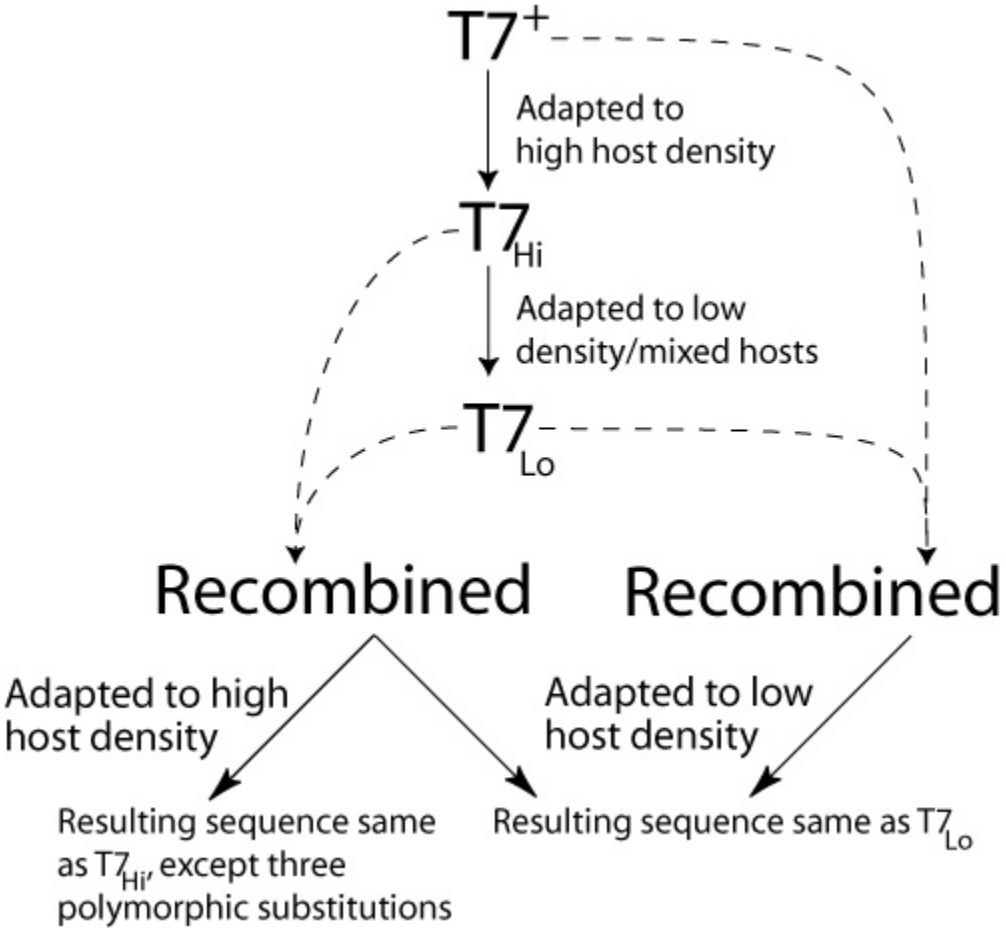


Figure 4.4. Lysis time adaptation. Lysis time (L) can be broken down into eclipse time (E) plus post-eclipse time ($L - E$). Optimal lysis time is predicted as approximately $E + X$, where $X = 1 / r$ plus a small correction for variance in lysis time from Appendix 1. For this reason, a line $L = E$ provides a useful baseline for comparison of these values. In all cases, the putative optimum is based on current fitness (r) and eclipse time, hence is subject to change as these traits adapt. (A) Not surprisingly, $T7^+$ lysis was not optimal in high host density conditions prior to adaptation. (B) After adaptation to high host density, lysis time approached the optimum; eclipse time also shortened. (C) After adaptation to low host density, evolved lysis time increased slightly but remained well short of the putative optimum. Surprisingly, eclipse time also increased in this selection. The optima in these figures are referred to as putative optima, because their values are based on the observed r rather than the r that would be achieved at the optimum ($\hat{r}$). The low density conditions used in these adaptations used a mix of permissive and non-permissive hosts (the *formal* low density adaptation).

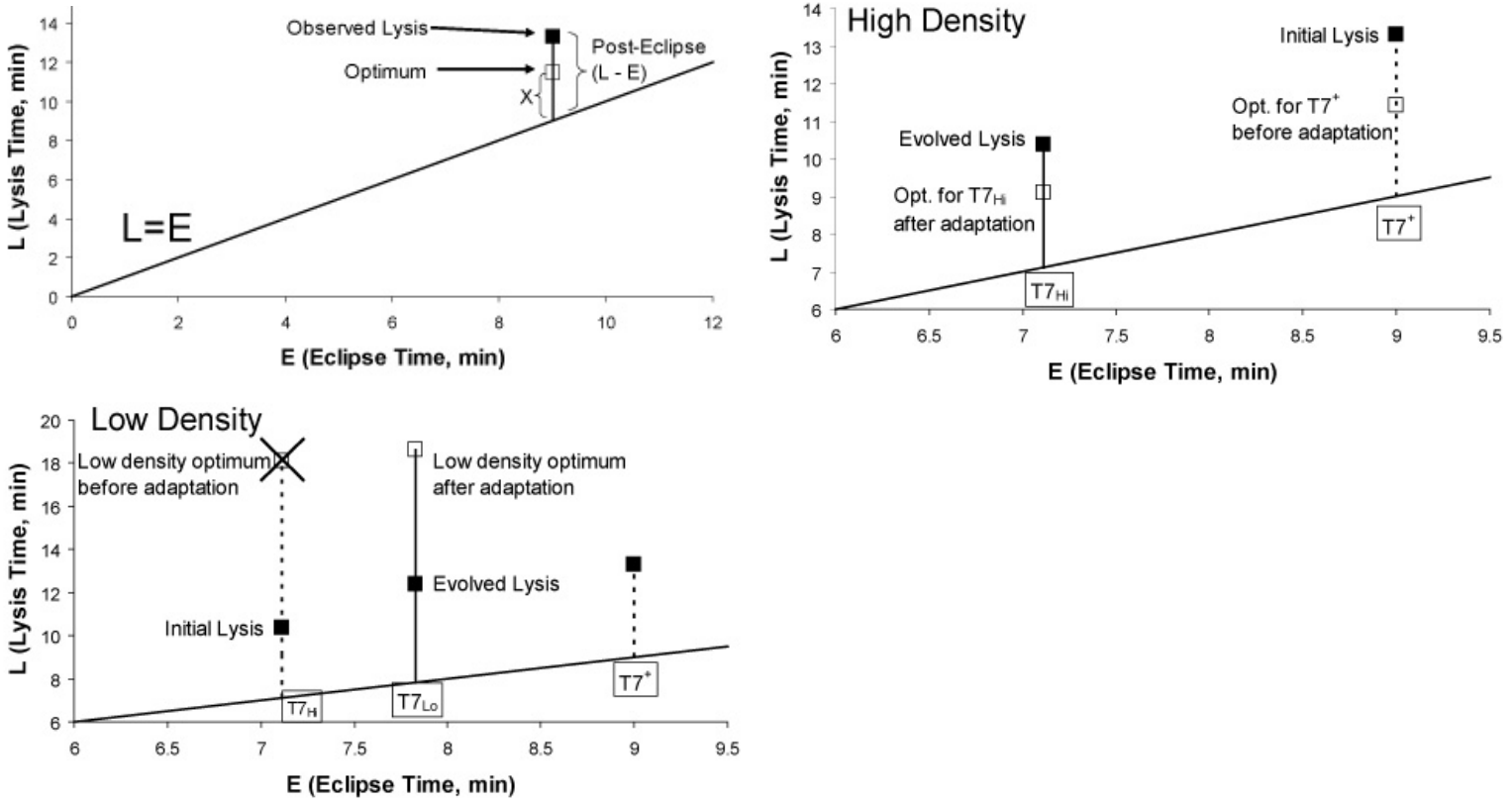


Figure 5.4. Strength of selection for the optimum. Equation (A1.d) was used to plot fitness against average lysis time (post-eclipse time) for parameters somewhat matching those of the high and low density adaptations. The optimum is at the peak, indicated by the filled circles. The vertical bars through the curves indicate a type of selective equivalence for 60 hr of adaptation: if a homogenous population's initial lysis time was at the boundary of this interval, a mutant whose lysis time was exactly optimal would ascend from an initial frequency of 10^{-6} to 0.5 in 60 hrs. Populations whose starting genotypes were inside this interval thus may not be able to evolve the optimal lysis time (in 60 hr of adaptation) even if the appropriate mutations arose. However, the strength of selection for the optimum is substantially greater at high host density than at low. Parameters were ($R = 80$, $c = 0.4$, $E = 7.1$, $\sigma^2 = 0.5$ for high density; $R = 70$, $c = 0.004$, $E = 7.8$, $\sigma^2 = 0.9$, with an additional term added to account for non-permissive hosts, from Bull 2006). No attempt was made to fit the observations quantitatively.

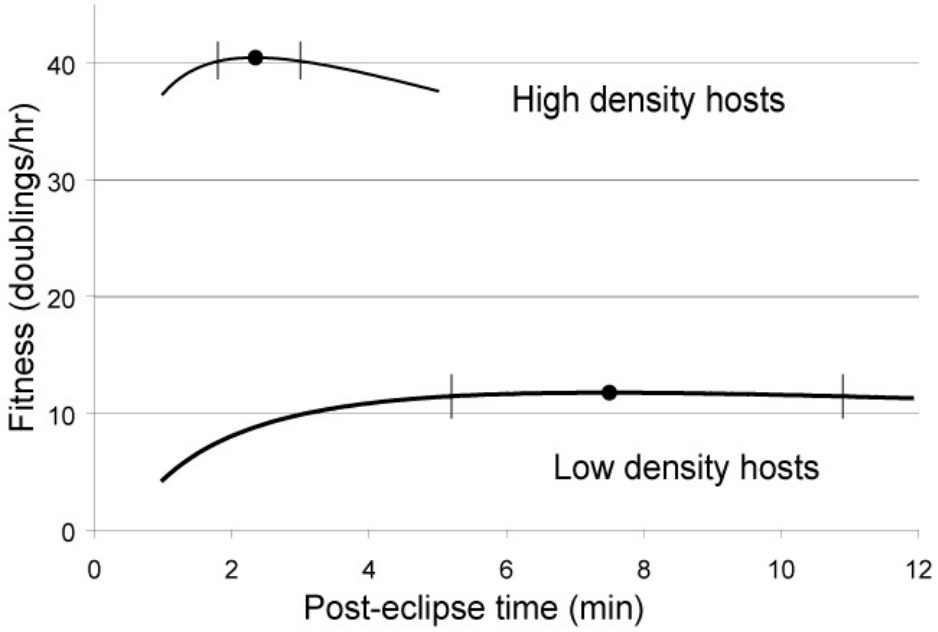


Table 1.2. T7 lines used in this study.

Phage Line	Description
$T7^{+}_{0}$	Wild-type T7.
$*T7^{+}_{61}$	$T7^{+}_{0}$ after adaptation to passaging conditions.
$AFK136_{0}$	T7 with mutant lysozyme, lacking lysis activity.
$*AFK136_{43}$	$AFK136_{0}$ after all experimental adaptation (43 passages)
$T7\Delta 3.5_{0}$	T7 deleted for lysozyme gene.
$T7\Delta 3.5_{22}$	$T7\Delta 3.5_{0}$ after 22 passages of adaptation.
$*T7\Delta 3.5_{62}$	$T7\Delta 3.5_{0}$ after all experimental adaptation (62 passages).
$T7\Delta 3.5,16_{Q89H}$	Phage $T7\Delta 3.5_{0}$ plus mutation 16_{Q89H} and a silent mutation at nucleotide 30654 T->G (16_{A20A}).
$AFK136_{swap}$	$AFK136_{0}$, with 16_{G14S}, 16_{Q89H}, 16_{N117K}, and 17_{T118A} from $T7\Delta 3.5_{62}$.
$T7^{+}_{swap}$	$T7^{+}_{0}$, with 16_{G14S}, 16_{Q89H}, 16_{N117K}, and 17_{T118A} from $T7\Delta 3.5_{62}$.
$T7\Delta 3.5_{62(A)}$	Isolate from $T7\Delta 3.5_{62}$ with 16_{G14S},16_{Q89H} and 16_{N117K} mutations. Genotype A in Fig. 3.2.
$T7\Delta 3.5_{62(B)}$	Isolate from $T7\Delta 3.5_{62}$ that is the same as $T7\Delta 3.5_{62(A)}$ except that it lacks $16_{G14S.}$ Genotype B in Fig. 3.2.
$*T7^{+}/\Delta 3.5_{62}$	Recombinant between $T7^{+}_{0}$ and $T7\Delta 3.5_{62}$, adapted to fix mutations of fittest genotype. Is 3.5^{+} and contains mutations $10B_{E375K}$, 16_{G14S}, 16_{Q89H} and 17_{T118A}.

* indicates lines whose sequences and phenotypes were analyzed as cultures. Genetic variants present only at low frequencies would not have been detected but might affect the phenotype of the culture.

Table 2.2. Phenotypic traits in T7 lines, with 95% confidence intervals.

Line	Fitness	Mean Lysis Time	Slope of lysis curve at mean[1]
$T7^{+}_{0}$	35.6 ± 0.4	14.4 ± 1.1	-0.36 ± 0.23
$T7^{+}_{61}$	41.9 ± 2.1	10.1 ± 0.1	-0.48 ± 0.16
$AFK136_{0}$	11.4 ± 1.1	24.6 ± 2.6	-0.02 ± 0.003
$AFK136_{43}$	35.4 ± 1.3	11.7 ± 0.6	-0.17 ± 0.19
$T7\Delta3.5_{0}$	10.9 ± 0.4	28.2 ± 5.5	-0.06 ± 0.05
$T7\Delta3.5_{8}$	12.0 ± 2.9	25.4 ± 4.2	-0.04 ± 0.02
$T7\Delta3.5_{22}$	23.0 ± 1.3	16.8 ± 5.3	-0.09 ± 0.06
$T7\Delta3.5_{62}$	32.4 ± 1.5	12.4 ± 0.6	-0.42 ± 0.08
$T7\Delta3.5{:}\mathit{16}_{Q89H}$	20.5 ± 2.9	15.9 ± 0.4	-0.24 ± 0.05
$AFK136_{swap}$	31.9 ± 3.3	13.1 ± 0.9	-0.26 ± 0.23
$T7^{+}_{swap}$	32.7 ± 1.0	12.9 ± 0.3	-0.36 ± 0.06
$T7\Delta3.5_{62(A)}$	ND	11.6 ± 0.8	-0.32 ± 0.14
$T7\Delta3.5_{62(B)}$	ND	12.4 ± 0.7	-0.34 ± 0.13

ND=Not determined

[1]A measure of lysis synchrony. Per minute decline in culture turbidity (as a proportion of total turbidity) measured at mean lysis time (see Materials and Methods). More negative values correlate with more abrupt lysis.

Table 3.2. Genetic evolution in adapted T7 lines relative to starting phage[1].

Nucleotide	Gene	Change	Gene Function	T7Δ*3.5*$_8$	T7Δ*3.5*$_{22}$	T7Δ*3.5*$_{62}$	AFK136$_{43}$	T7$^+$/Δ*3.5*$_{62}$	T7$^+_{61}$
5550	*1*	A->G T794A	T7 RNAP	+	+	+	ND	-	-
7964	*1.6*	G->A R20H	Unknown	-	+	+	ND	-	-
24088* [2]	*10B*	G->A E375K	Minor Coat Protein	-	-	+	ND	+	+
24171	Tø	C deletion	T7 RNAP Terminator	-	-	+	ND	-	-
30634*	*16*	G ->A G14S	Internal Core Protein	-	-	+/- [3]	-	+	-
30646	*16*	A->C K18Q	"	-	-	-	+/-	-	-
30660	*16*	C->A silent	"	-	-	-	+/-	-	-
30701	*16*	C->T T36I	"	-	-	+/-	-	-	-
30860	*16*	A->T Q89L	"	-	-	-	+/-	-	-
30861*	*16*	A->C Q89H	"	-	+	+/-	+/-	+	+
30945	*16*	T->G N117K	"	-	-	+/-	+/-	-	-
34975*	*17*	A->G T118A	Tail Fiber	-	-	+	ND	+	+
1257-2736	*0.3-0.7* fusion *0.4-0.6* deletion	Deletion	Various[4]	-	-	-	ND	ND	+
15094	*5*	G->T A248S	DNA Polymerase	-	-	-	ND	ND	+
29770	*15*	G->A K482K	Internal Core Protein	-	-	-	ND	ND	
32860	*16*	G->A G756S	"	-	-	-	ND	ND	+
36389	*17.5*	A->G I16V	Holin	-	-	-	ND	ND	+/- [5]
36492	*17.5*	T->G I50S	"	-	-	-	ND	ND	+/- [5]

ND=Not determined.

[1] Mutations below the dotted line were found only in T7$^+_{61}$.

[2] * Indicates mutations present in T7$^+$/Δ*3.5*$_{62}$ and therefore possibly non-compensatory.

[3] +/- Indicates polymorphism in lysate.

[4] Deletion H1 of Studier (1973). Retains anti-restriction activity of *0.3*, and transcription shut-off but not protein kinase activity of *0.7*. Genes *0.4 - 0.6* have no known function.

[5] All six T7$^+_{61}$ purified phages contained one gene *17.5* mutation. Two contained *17.5*$_{I16V}$, four contained *17.5*$_{I50S}$.

Table 1.3. Genetic evolution of adapted lines.

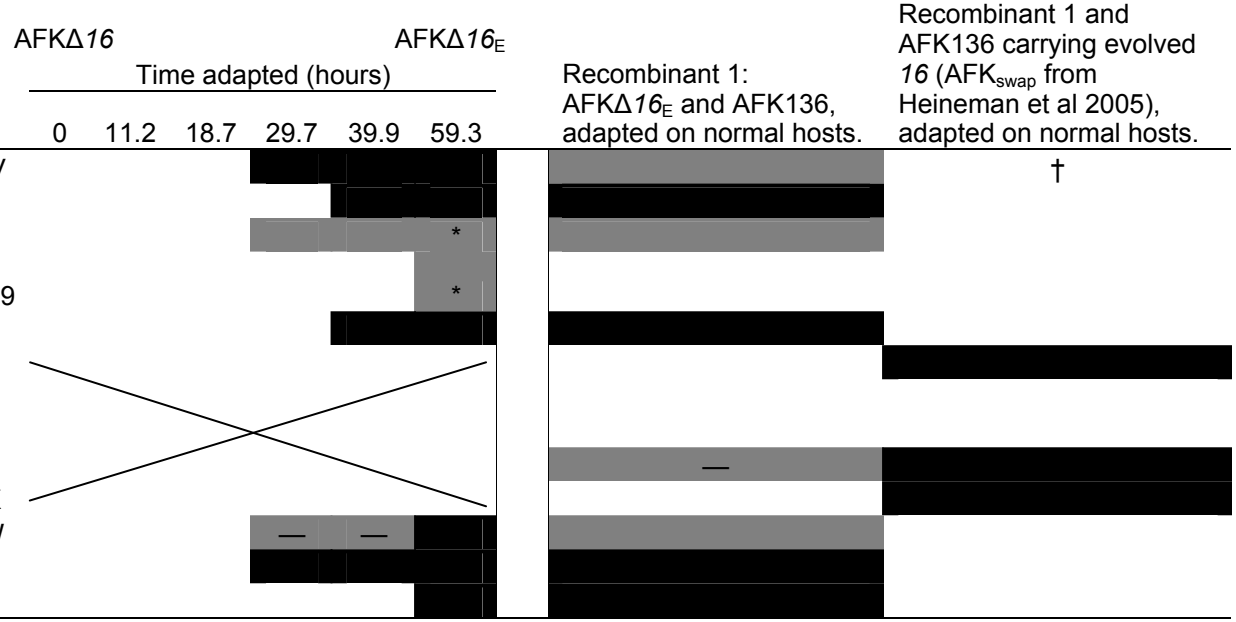
Gene	Nucleotide	Change	Gene Function	$T7^{+}_{E}$	$T7\Delta 3.5_{E}$	$AFK136_{E}$	$T7\Delta 17.5_{E}$	$T7\Delta 3.5\Delta 17.5_{E}$	$AFK\Delta 16_{E}$
Removes genes *0.3-0.6* entirely, and most of *0.7*[†]	575-2742	Deletion	Various						
Fusion between 59th codon of *0.3*, 187th codon of *0.7*[‡]	1101-2583	Deletion	Various						
0.3-0.7 fusion, *0.4-0.6* deletion[‡]	1257-2736	Deletion	Various						
0.7	2606	A->G M196V	Protein kinase						
Immediately before *1*	3163	G->A							
1	5274	G->A A702T	T7 RNA polymerase						
1	5550	A->G T794A	T7 RNA polymerase						
1.2	6227-8	T,G deleted	Non-essential, F exclusion						
1.6	7964	G->A R20H	Unknown						
1.6	8027	T->G L41W	Unknown						
1.8	8750	T->C M1T	Non-essential						*
1.8	8768	C insert, missense at 9th codon	Non-essential						*
3.5 just prior to deletion	10718	C->T stop at 5th codon							
3.8	11294	G del, nonsense at 24th codon	Non-essential						
4.3	13385	C->T L12L	Nonessential						
5	15094	G->T A248S	DNA polymerase					i	
6.3	18458	G->A G22D	Non-essential						
10B	24088	G->A E375K	Minor coat protein					i	
Tφ	24171	C deletion	T7 RNAP terminator						
Tφ	24178	G deletion	T7 RNAP terminator						
12	26153	A->G N438D	Tail protein						

Table 1.3 continued.

Gene	Nucleotide	Change	Gene Function	$T7^{+}_{E}$	T7Δ*3.5*$_{E}$	AFK136$_{E}$	T7Δ*17.5*$_{E}$	T7Δ*3.5*Δ*17.5*$_{E}$	AFKΔ*16*$_{E}$
15	29770	G->A K482K	Internal core protein					i	
16	30634	G->A G14S	Internal core protein						
16	30642	C->T F16F	Internal core protein						
16	30646	A->C K18Q	Internal core protein						
16	30660	C->A A22A	Internal core protein						
16	30701	C->T T36I	Internal core protein						
16	30860	A->T Q89L	Internal core protein						
16	30861	A->C Q89H	Internal core protein					i	
16	30945	T->G N117K	Internal core protein					i	
16	31819	G->A A409T	Internal core protein						
16	32860	G->A G756S	Internal core protein					i	
17	34975	A->G T118A	Tail fiber						
17	35438	T->G L272W	Tail fiber						
17.5	36350	T->C S3P	Holin						
17.5	36389	A->GI16V	Holin	*					
17.5	36492	T->G I50S	Holin	*					
19.5	39459	C->A A24D	Non-essential						
19.5	39501	A->G E38G	Non-essential						

Changes are marked in black. Polymorphic changes marked in grey.
i indicates substitution present in initial phage
* indicates that every isolate sampled from population had either one or the other of these substitutions.
† Gene *0.7* is unlikely to be translated..
‡ Should retain protein kinase activity of *0.7* (Studier 1973).

Table 2.3. Genetic evolution of AFKΔ*16* at intermediate times for adaptation and after recombination assays.



Gene	Nucleotide	Change
0.7	2606	A->G M196V
1.6	8027	T->G L41W
1.8	8750	T->C M1T
1.8	8768	C insert, missense at 9
6.3	18458	G->A G22D
16	30634	G->A G14S
16	30660	C->A silent
16	30860	A->T Q89L
16	30861	A->C Q89H
16	30945	T->G N117K
17	35438	T->G L272W
17.5	36350	T->C S3P
19.5	39501	A->G E38G

Changes are marked in black. Polymorphic changes marked in grey.
Intermediate and recombinant phages were sequenced from the population and only over regions containing substitutions in AFKΔ*16*$_E$.
Large X indicates gene *16* sites not present in these lines due to gene deletion.
— indicates the substitution was only present at very low levels.
* indicates that every isolate sampled from population had either one or the other of these substitutions.
† instead has deletion of bases 1253-2741, inclusive. Gene *0.3-0.7* fusion as Studier (1973), but with three extra residues remaining.

Table 1.4. Phenotypic traits of T7 lines, with standard errors (computed from the observations) and number of assays.

	$T7_{Hi}$	$T7_{Lo}$
Optimum (high density) †	9.1	10.1
Optimum (low density/mixed) †	18.1	18.6
Lysis time †	10.4 ± 0.2 (3)	12.4 ± 0.0 (2)
Lysis variance €	0.4 ± 0.2 (3)	0.9 ± 0.2 (2)
High moi lysis time †	10.5 ± 0.2 (6)	11.8 ± 0.1 (6)
Eclipse time †	7.1 ± 0.1 (4)	7.8 ± 0.2 (3)
Burst size	266 ± 16 (6)	327 ± 27 (7)
Fitness (high density) ‡	47.9 ± 0.4 (4)	43.7 ± 1.2 (3)
Fitness (low density) ‡	12.1 ± 0.5 (4)	11.7 ± 0.4 (3)
Fitness (low density/mixed) ‡	7.9 ± 0.6 (4)	8.1 ± 0.8 (4)
Adsorption £	3.3 ± 0.3 (2)	4.0 ± 0.4 (3)

† minutes, € minutes^2, ‡ doubling/hour, £ 10^{-9} ml/min.

r = Fitness x 0.011552

Optima estimated as $\overline{E} + 1/\overline{r} + \overline{r}\overline{\sigma}^2 - (5/3)\overline{r}^3\overline{\sigma}^4$ (Appendix 1).

Table 2.4. Genetic evolution in $T7_{Hi}$ and $T7_{Lo}$.

Nucleotide	Gene	Change	Gene function	$T7_{Hi}$	$T7_{Lo}$
1257-2736	*0.3-0.7* fusion, *0.4-0.6* deletion	Deletion	Various[a]	+	+
15094	*5*	G->T A248S	DNA Polymerase	+	+
24088	*10B*	G->A E375K	Minor coat protein	+	+
30861	*16*	A->C Q89H	Internal core protein, lysin activity in some conditions	+	+
30945	*16*	T->G N117K	Internal core protein, lysin activity in some conditions	+	+
32860	*16*	G->A G756S	Internal core protein, lysin activity in some conditions	+	+
34975	*17*	A->G T118A	Tail fiber	+	+
36492	*17.5*	T->G I50S	Holin	+	+
4325	*1*	C->T Y385Y	T7 RNA polymerase	-	+[b]
5451	*1*	A->G I761V	T7 RNA polymerase	-	+
6238	*1.2*	C->T A34A	Non-essential, F exclusion, inhibits *E. coli* deoxyguanosine triphosphohydrolase.	-	+[b]
13481	*4.3*	C->T L44L	Non-essential, unknown.	-	+[b]
31735	*16*	G->A A381T	Internal core protein, lysin activity in some conditions	-	+
32142	*16*	G->A E516E	Internal core protein, lysin activity in some conditions	-	+
36320	Intergenic, 24 bp before *17.5*	G->A	Upstream of ribosome-binding sequence of holin	-	+

Mutations below the horizontal line were new in $T7_{Lo}$. Mutations above the line evolved from $T7^{+}$ to $T7_{Hi}$.

[a]Deletion H1 of Studier (1973). Retains anti-restriction activity of *0.3* and transcription shut-off but not protein kinase activity of *0.7*. Genes *0.4-0.6* have no known function.
[b]Substitutions polymorphic in T7 recombinant adapted to high density, indicating that they are not strongly disadvantageous at high host density.

Appendices

APPENDIX 1. ENVIRONMENTAL VARIATION IN LYSIS TIME.

The optimality model developed in Bull 2006 assumed that the lysis time (L) of a genotype is constant across hosts. Measurements of lysis time from phages indicate that lysis is not synchronous and thus that L is not necessarily constant (variation in lysis time could, in principle, stem from variation in time to adsorption). The optimality model was therefore modified to consider environmental variation in L. The model adopted here assumed that (i) lysis time, L, $= \mathrm{E} + x$, where x is the post-eclipse time, distributed with mean μ and variance σ^2. Burst size (b) follows the linear model $b = Rx$, with R being the constant rate of intracellular phage accumulation. From Bull (2006), the intrinsic growth rate (r) of the phage population satisfies

$$r = (b\mathrm{e}^{-Lr} - 1)c = (Rx\mathrm{e}^{-r(E+x)} - 1)c \qquad \text{(A1.a)}$$

L and b are invariant (c is the product of cell density times adsorption; for our experimental conditions, we neglect the phage death rate and washout terms in Bull 2006). When x varies among infections within a culture, the relevant equation is

$$r = E_x\{b\mathrm{e}^{-Lr} - 1\}c = [\mathrm{Re}^{-rE}\, E_x(x\mathrm{e}^{-rx}) - 1]c \qquad \text{(A1.b)}$$

where E_x is the expectation taken over x When x follows a normal distribution with mean μ and variance σ^2, (A1.b) becomes

$$r = \mathrm{R}\, c\, \mathrm{e}^{-rE}(\mu - r\sigma^2)e^{-r\mu+\frac{r^2\sigma^2}{2}} - c \qquad \text{(A1.c)}$$

Assuming normality has the advantage of allowing an exact analytic solution for the optimum (below). However, normality of x allows negative values, which are biologically disallowed. Thus, it is also useful to consider a distribution confined to x>0, such as a gamma. When x follows a gamma distribution, (A1.b) becomes

$$r = \mathrm{R}\, c\, \mathrm{e}^{-rE}\frac{\mu}{\left(1 + r\frac{\sigma^2}{\mu}\right)^{\frac{\mu^2}{\sigma^2}+1}} - c \qquad \text{(A1.d)}$$

For each model, the optimum lysis time is found by differentiating the respective equation (A1.c or A1.d) with respect to x and setting $\frac{\partial r}{\partial x} = 0$. Differentiating (A1.c) leads to text equation (1b) as an exact solution. Differentiating (A1.d) leads to

$$1 + \hat{r}\hat{\mu} + 2\hat{r}\frac{\sigma^2}{\hat{\mu}} - 2\hat{\mu}\left(\hat{r} + \frac{\hat{\mu}}{\sigma^2}\right)\ln\left(1 + \hat{r}\frac{\sigma^2}{\hat{\mu}}\right) = 0 \qquad \text{(A1.e)}$$

Approximations.

A normal distribution allows *x* to take on negative values, and the model, in turn, assigns negative fecundity. Consequently, text equation (1b) may not be accurate for large variances. To establish bounds on σ^2 that will avoid negative values, note that if $\mu = 2\sigma^2$ for a normal distribution, then less than 1% of values are negative (if $\mu = \sigma^2$ then 16% are negative). Furthermore, the optimum approximately satisfies $\hat{\mu} = \frac{1}{\hat{r}}$, so $r\sigma^2 < 1/2$ should conservatively ensure that (1b) is accurate, and $r\sigma^2 < 1$ may be adequate. However, this correction amounts to at most a minute increase in the optimum, and a minute may be well within the bounds of measurement error for the other terms in the optimum. The gamma distribution does not pose the problem of negative values, but an analytic solution of (A1.e) is not attainable. An approximate solution is

$$\hat{u} = \frac{1}{r} + r\sigma^2 - \frac{5}{3}r^3\sigma^4 \qquad \text{(A1.f)}$$

but this approximation is useful only if $r\sigma^2 < 1$ and $r^2\sigma^2 < 0.16$. However, (A1.e) can be solved numerically for arbitrary *r* and σ^2.

APPENDIX 2. DISTRIBUTION OF LYSIS TIMES.

The model of infection times is as follows. In a culture of cells to which phage have been added at time 0, the time to lysis since phage addition is $T = X + Y$, where X is the time to adsorption and Y is the time to lysis following adsorption. The variable X is known to follow an exponential distribution with parameter A, the product of cell density and adsorption (Adams 1959). Let the distribution of Y be $f(y)$, with y strictly positive.

To obtain the cumulative probability of lysis to time t, $P(t)$, we want $P(t) = \Pr(T \le t) = \Pr(X + Y \le t) = \Pr(X \le t - Y)$. From this and the assumption that adsorption follows an exponential distribution with parameter A, it follows that

$$\begin{aligned} P(t) &= \int_0^t f(y) \int_0^{t-y} A\mathrm{e}^{-Ax}\,dx\,dy \\ &= \int_0^t (1 - \mathrm{e}^{-A(t-y)}) f(y)\,dy \end{aligned} \qquad \text{(A2.a)}$$

For appropriate choice of $f(y)$, it may be possible to solve (A2.a). However, (A2.a) lends itself to numerical integration, which is the approach adopted here. For this study, we used a gamma pdf for $f(y)$.

Assays of lysis time are usually conducted by diluting the culture after a moderate interval of adsorption but before any observable lysis (thus preventing secondary infections). Assuming that further adsorption is stopped at time $t = \tau$, equation (A2.a) is easily modified to

$$P(t) = \frac{\int_0^{t-\tau} (e^{-A\tau} - e^{-A(t-y)}) f(y) dy}{1 - e^{-A\tau}} \qquad \text{(A2.b)}$$

Finally, if our model is L = E + t, where E is fixed, the distribution of post-eclipse times (y) is obtained by translating total lysis time by E and fitting data to the variable t = L – E.

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