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Evolutionary Robustness of an Optimal Phenotype: Re-evolution of Lysis in a Bacteriophage Deleted for Its Lysin Gene

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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. Phage 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 lysis more rapid or delayed than this optimum 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 that violated model assumptions.

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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 that varies with host density and host physiology (Abedon et al. 2001, 2003; Wang et al 1996; Wang ms in prep), 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 that, 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 were extensive and 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 R.H.H. and I.J.M.), 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, 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 suboptimal 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).

Materials and 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: (1) 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), (2) T7Δ*3.5*, lacking all but the first six codons of the 152 amino acid lysozyme gene *3.5* (Zhang and Studier 2004); and (3) 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 $T7\Delta3.5_8$. 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*] (Yang and Richardson 1997) was used for transfections of T7 genomic DNA. 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 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°C in an orbital water bath (200 rpm) and allowed to grow for 1 h to a density of $1–2 \times 10^8$/ml, at which point 10^4–10^7 phage were added from a variable volume less than 200 μl. The culture was then incubated for 20–60 min, 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, $T7\Delta3.5_0$ (the original stock of T7Δ*3.5*) was passaged for 43 h across 62 flasks to yield $T7\Delta3.5_{62}$. T7 $AFK136_0$ was passaged for 25.5 h 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 ($T7\Delta3.5_{54}$) 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_t is the number of phage in the flask at time t 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 $1.0\text{-}\phi(\mu,\sigma^2,t)$, 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). 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 min to decrease further adsorption. We then titered phage from samples at various time points, before and

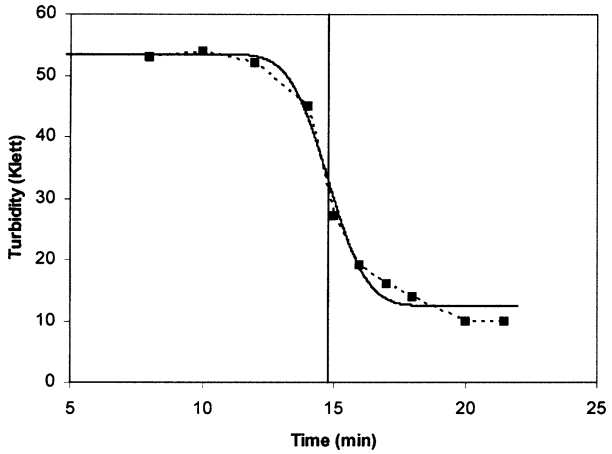

Fig. 1. Lysis curve for $T7^{+}{}_{0}$. The dotted line shows a representative lysis curve based on Klett values. The solid curve shows the best-fit cumulative normal curve, and the vertical line shows the estimated mean lysis time for these data.

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 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 (see Fig. 6C), 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 min).

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 (1) $AFK136_{swap}$, which is $AFK136_0$ plus four mutations from $T7\Delta3.5_{62}$: 16_{G14S}, 16_{Q89H}, 16_{N117K}, and 17_{T118A}; (2) $T7^{+}{}_{swap}$, which has the same four mutations of (1) but in a $T7^{+}{}_{0}$ genetic background; and (3) $T7\Delta3.5,16_{Q89H}$ (from an *Mlu* I fragment swap between $T7^{+}{}_{0}$ and $T7\Delta3.5_{22}$), which differs from $T7\Delta3.5_0$ by the presence of the gene 16_{Q89H} mutation and that was sequenced to ensure a lack of gene *1* mutations (Table 1).

Table 1. T7 lines used in this study

Phage Line	Description
$T7^{+}{}_{0}$	Wild-type T7.
$T7^{+}{}_{61}$[a]	$T7^{+}{}_{0}$ after adaptation to passaging conditions.
$AFK136_0$	T7 with mutant lysozyme, lacking lysis activity.
$AFK136_{43}$[a]	$AFK136_0$ after all experimental adaptation (43 passages).
$T7\Delta3.5_0$	T7 deleted for lysozyme gene.
$T7\Delta3.5_{22}$	$T7\Delta3.5_0$ after 22 passages of adaptation.
$T7\Delta3.5_{62}$[a]	$T7\Delta3.5_0$ after all experimental adaptation (62 passages).
$T7\Delta3.5,16_{Q89H}$	Phage $T7\Delta3.5_0$ 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 $T7\Delta3.5_{62}$.
$T7^{+}{}_{swap}$	$T7^{+}{}_{0}$, with 16_{G14S}, 16_{Q89H}, 16_{N117K} and 17_{T118A} from $T7\Delta3.5_{62}$.
$T7\Delta3.5_{62(A)}$	Isolate from $T7\Delta3.5_{62}$ with 16_{G14S}, 16_{Q89H} and 16_{N117K} mutations. Genotype A in Fig. 3.
$T7\Delta3.5_{62(B)}$	Isolate from $T7\Delta3.5_{62}$ that is the same as $T7\Delta3.5_{62(A)}$ except that it lacks 16_{G14S}. Genotype B in Fig. 3.
$T7/\Delta3.5_{62}$[a]	Recombinant between $T7^{+}{}_{0}$ and $T7\Delta3.5_{62}$, adapted to fix mutations of fittest genotype. Is 3.5^{+} and contains mutations $10B_{E375K}$, 16_{G14s}, 16_{Q89H} and 17_{T118A}.

[a]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.

Results

Adaptations to compensate for lost lysozyme functions were carried out in two phage lines; $T7\Delta3.5$, 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 for strain details).

Phenotypic Evolution of Lysis Time and Fitness

On host IJ1133 at 37°C, $T7^{+}{}_{0}$ had a fitness of 35.6 doublings/h, 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 ($T7\Delta3.5_0$) was only 10.9 doublings/h, while lysis was delayed to 28.2

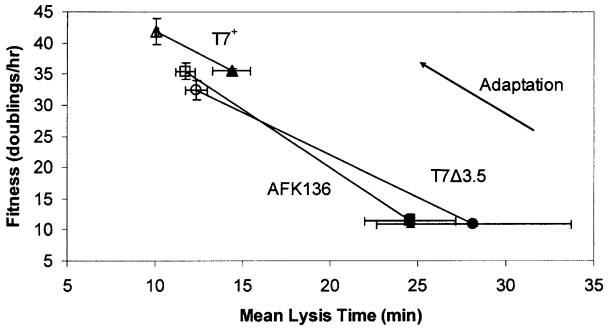

Fig. 2. Fitness and lysis time adaptation. Solid symbols represent initial values, open symbols represent values after adaptation. T7Δ*3.5*: ○, ●; AFK136: □, ■; $T7^+$; Δ, ▲. Each line represents the adaptation of a phage line. In all lines, lysis time decreased and fitness increased during adaptation, 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.

Table 2. Phenotypic traits in T7 lines, with 95% confidence intervals

Line	Fitness	Mean lysis time	Slope of lysis curve at mean[a]
$T7^+{}_0$	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
T7Δ*3.5*$_0$	10.9 ± 0.4	28.2 ± 5.5	−0.06 ± 0.05
T7Δ*3.5*$_8$	12.0 ± 2.9	25.4 ± 4.2	−0.04 ± 0.02
T7Δ*3.5*$_{22}$	23.0 ± 1.3	16.8 ± 5.3	−0.09 ± 0.06
T7Δ*3.5*$_{62}$	32.4 ± 1.5	12.4 ± 0.6	−0.42 ± 0.08
T7Δ*3.5*,*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
T7Δ*3.5*$_{62(A)}$	ND	11.6 ± 0.8	−0.32 ± 0.14
T7Δ*3.5*$_{62(B)}$	ND	12.4 ± 0.7	−0.34 ± 0.13

ND = not determined.

[a]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.

min and highly asynchronous (Fig. 2, Table 2). $AFK136_0$ was similarly affected by loss of just the amidase activity of lysozyme, with fitness reduced to 11.4 doublings/h 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, Table 2). Fitness also improved, evolving to 32.4 and 35.4 doublings/h 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). 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°C (Studier 1969). Indeed, considerable adaptation of $T7^+$ to the passaging conditions occurred, from 35.6 to 41.9 doublings/h, while lysis time also shortened, from 14.4 to 10.1 min (Fig. 2, Table 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). 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 personal communication). 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 h 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

Table 3. Genetic evolution in adapted T7 lines relative to starting phage

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[a]	*10B*	G->A E375K	Minor coat protein	–	–	+	ND	+	+
24171	Tø	C deletion	T7 RNAP terminator	–	–	+	ND	–	–
30634[a]	*16*	G->A G14S	Internal core protein	–	–	+/–	–	+	–
30646	*16*	A->C K18Q	Internal core protein	–	–	–	+/–	–	–
30660	*16*	C->A silent	Internal core protein	–	–	–	+/–	–	–
30701	*16*	C->T T36I	Internal core protein	–	–	+/–	–	–	–
30860	*16*	A->T Q89L	Internal core protein	–	–	–	+/–	–	–
30861[a]	*16*	A->C Q89H	Internal core protein	–	+	+/–	+/–	+	–
30945	*16*	T->G N117K	Internal core protein	–	–	+/–	+/–	–	–
34975[a]	*17*	A->G T118A	Tail fiber	–	–	+	ND	+	+
1257–2736	*0.3–0.7* fusion *0.4–06* deletion	Deletion	Various[b]	–	–	–	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	Internal core protein	–	–	–	–	ND	+
36389	*17.5*	A->G I16V	Holin	–	–	–	ND	ND	+/–[c]
36492	*17.5*	T->G I50S	Holin	–	–	–	ND	ND	+/–[c]

ND = not determined. Mutations below the line were found only in T7$^+{}_{61}$. +/– Indicates polymorphism in lysate.
[a]Indicates mutations present in T7$^+$/Δ*3.5*$_{62}$ and therefore possibly non–compensatory.
[b]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.
[c]All six T7$^+{}_{61}$ isolates contained one gene *17.5* mutation. Two contained *17.5*$_{I16V}$, four contained *17.5*$_{I50S}$.

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) 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 [a] in Table 3) 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.

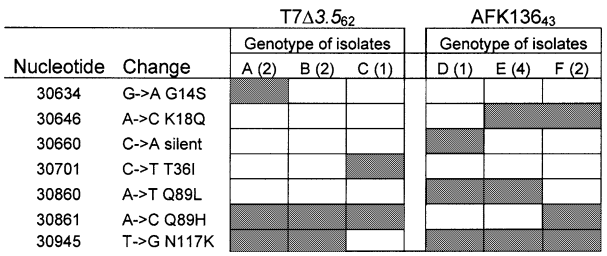

Fig. 3. Gene *16* mutations found in individual phage genomes obtained from T7Δ*3.5*$_{62}$ and AFK136$_{43}$ lysates. The 5' end of gene *16* was sequenced using phages from several isolated plaques in order to identify polymorphisms. A filled rectangle means that the mutation was present in an individual 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.

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), 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, compensated 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, some phages from the polymorphic T7Δ*3.5*$_{62}$

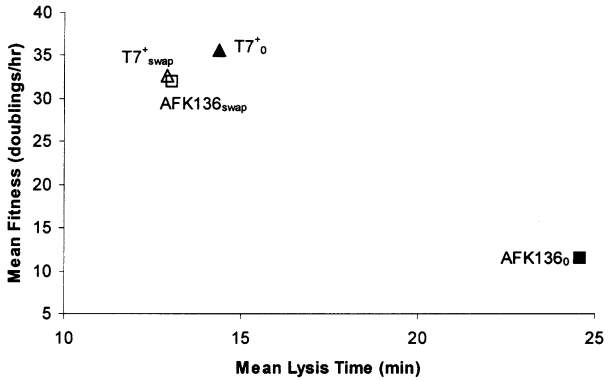

Fig. 4. Apparent stabilizing selection for lysis time based on fitness and lysis time of mutants thought to differ primarily in lysis mechanisms. In the absence of gene *16* mutations, lysozyme deficiency has a large effect on fitness and lysis time, which is largely rectified by gene *16* mutations. Fitness at an intermediate lysis time is higher than at more extreme times, suggesting an intermediate optimum. This may actually be caused largely by violations of the optimality model.

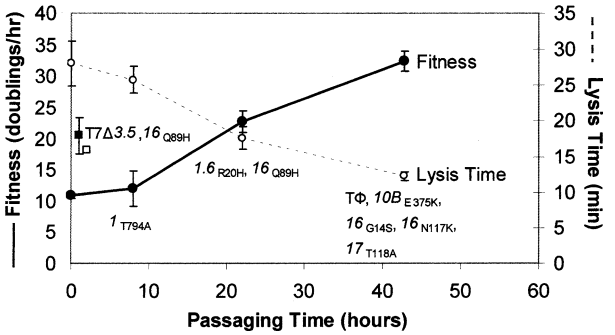

Fig. 5. Phenotypic evolution and appearance of mutations during T7Δ*3.5* evolution. The horizontal axis indicates the times (hours of passage) during the adaptations when phage lysates were obtained, the data points correspond to passage numbers 0, 8, 22, and 62. Fitness (closed circles) and lysis time (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.

lysate were found which differed in the presence (genotype A, Fig. 3) 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 min instead of 12.4 min (Table 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$^+$$_{swap}$ and AFK136$_{swap}$, Table 1). Both phages also carry a gene *17* mutation that is not thought to affect lysis. AFK136$_{swap}$ had a fitness of 31.9 doublings/h (Table 2), much higher than AFK136$_0$ (11.4 doublings/h; $p < 0.0001$; two-tailed *t*-test) and a dramatically reduced lysis time (12.9 min instead of 26.5, $p < 0.0001$; two-tailed *t*-test, Fig. 4). However, both the fitness and lysis time of AFK136$_{swap}$ and T7$^+$$_{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; 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). The gene *16*$_{Q89H}$ mutation has a major influence on both lysis time and fitness in T7Δ*3.5*, increasing fitness by 10 doublings/h and speeding lysis by 12 min (Fig. 5 and Table 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 rate or 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, Fig. 6).

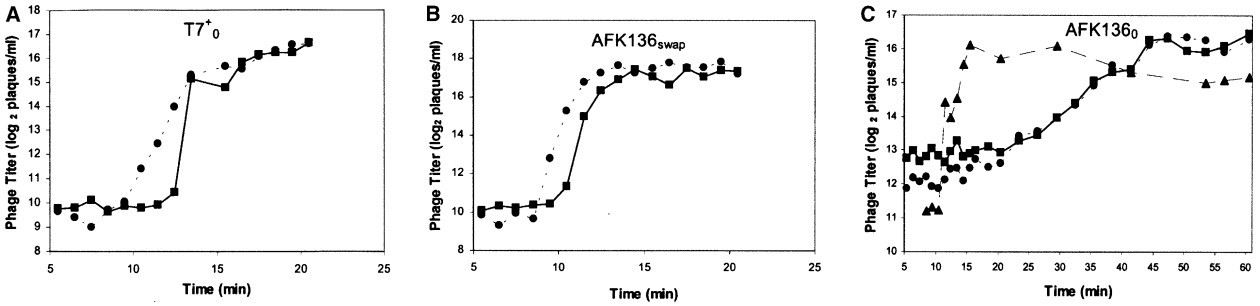

Fig. 6. Phage release for $T7^{+}_{0}$ (**A**), $AFK136_{swap}$ (**B**), and $AFK136_{0}$ (**C**). Phage titer of samples treated with chloroform (circles) or untreated (squares) at various time points after infection. A different replicate of $AFK136_{0}$ (triangles) was also subjected to artificial 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.

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. 6C). Artificial lysis by the addition of lysozyme plus EDTA, followed by chloroform, indicates that most phage production occurs by 15.5 min, 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 min 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). 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, 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 $T7\Delta3.5_{62}$ 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 $T7\Delta3.5_{0}$ and $T7\Delta3.5_{8}$, the latter carrying the gene *1* mutation, with respective fitnesses of 10.9 and 12.0 doublings/h and lysis times of 28.2 and 25.4 min (Table 2, Fig. 5). 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 threefold increase in intracellular phage results (Lyakhov et al. 1997; Zhang and Studier 1995, 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 $T7\Delta3.5_{0}$ 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 $T7\Delta3.5,16_{Q89H}$ was, therefore, compared directly to $T7\Delta3.5_{22}$. Both phages carry the 16_{Q89H} mutation, which shortens the time of lysis. However, $T7\Delta3.5,16_{Q89H}$ lacks the 1_{T794A} mutation of $T7\Delta3.5_{22}$ ($T7\Delta3.5,16_{Q89H}$ 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, $T7\Delta3.5_{22}$ should have a much higher fitness than $T7\Delta3.5,16_{Q89H}$. In contrast to this expectation, $T7\Delta3.5_{22}$ has a fitness only 2.5 doublings/h higher than $T7\Delta3.5,16_{Q89H}$ ($p < 0.03$; two-tailed *t*-test, Fig. 5). This difference was not significantly greater than that between $T7\Delta3.5_{0}$ and $T7\Delta3.5_{8}$ ($p < 0.25$) by a 4-way two-tailed *t*-test (Bull et al. 2000). Gene *1* was sequenced in an isolate from the end of each $T7\Delta3.5,16_{Q89H}$ 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/h 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 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 gp*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, b; Nakagawa et al. 1985). $T7^{+}{}_{0}$ does not exhibit lysis-from-without and preliminary data (not shown) suggests that $T7^{+}{}_{\text{swap}}$ 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 mutations. Phages carrying 16_{Q89H} 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 $\text{T7}\Delta 3.5_{62}$ 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).

If a T7 ancestor lacked a lysozyme gene, and gp16 acted both at the initial stages of infection and as an endolysin, 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. However, the enzyme activity in SP6 and K1-5 virions is different from that in T7 (lysozyme vs. lytic transglycosylase) and the activity is fused to

different virion proteins, so virion-associated muralytic activity may have been acquired after the T7 and SP6 phage groups separated during evolution (Moak and Molineux 2004).

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 shows (Abedon et al. 2003), especially λ (Wang ms in prep). 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).

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 mutations observed in genes *3.5* and *16* are thought to primarily affect lysis. 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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