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Experimental Evolution of Viral Control over Host Lysis Timing

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Dillon Main Dorsey

Committee in charge:

Justin Meyer, Chair

Joseph Ecker

Katherine Petrie

2019

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The Thesis of Dillon Main Dorsey is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California San Diego

2019

DEDICATION

In recognition of the unending years of support and joy, our grand adventures around the world, the quiet peace I find when we are together, and for giving me all the love in the universe, this manual is dedicated to my fiancée Kay and our dog Lily.

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ABSTRACT OF THE THESIS

Experimental Evolution of Viral Control over Host Lysis Timing

by

Dillon Main Dorsey

Master of Science in Biology

University of California San Diego, 2019

Professor Justin Meyer, Chair

When a gene experiences new selective pressures from its environment, adaptative mutations may arise that alter its amino acid sequence that produce a protein better suited to the new pressure, or through mutations that tune the gene's expression to adjust to the new environment. Here we report evolution experiments to understand the second mode of adaptation by studying the evolution of λ, a bacterial virus or bacteriophage that infects *E. coli,* under controlled laboratory settings. We pressured λ to alter its lysis timing, which is controlled by a

well-studied gene regulatory system, primarily driven by the *S* gene. For 10 days, we pressured λ towards shortened or lengthened lysis times and found that λ evolved shorter lysis times repeatedly in both treatments. Lysis time evolved in most replicates without evolving cis-regulatory S mutations, suggesting more genes are involved in lysis timing circuit than previously reported. We found a single S regulatory mutation among 12 experimental populations. This mutation accounted for some of the fitness gains λ evolved. The mutation also increased the stochasticity of lysis timing, which we hypothesize helped increase phenotypic variance and allowed λ to adapt to changing pressures. Altogether we found that λ rapidly adapts to selective pressure on lysis timing, but in unintuitive ways. Clearly a more sophisticated understanding how gene regulation networks respond to evolutionary pressure is required to predict adaptation.

Introduction

As molecular biology research has progressed, researchers have learned more about the structure and function of DNA, how mutations cause heritable change, and how these changes help populations adapt to their environments. Moreover, in some cases researchers have constructed detailed models of how genotypes are expressed through the central dogma of molecular biology into phenotypes, and how the expression of genes and these phenotypes can depend on developmental timing and change in response to fluctuations in environmental conditions (Wittkopp, 2013, p. 413). There have even been significant steps taken to predict how natural selection will shape phenotypic evolution. For example, quantitative genetics studies have shown that measurements of natural selection and trait heritability can be used to predict how populations respond to evolutionary pressure over short periods of time (Falconer and Mackay 1996). Other phenotypic selection theories have even provided the basis for predicting how populations evolve over a long period of time in response to environmental changes (Stern and Orgozozo 2008). However, predicting the precise genetic mutations and regulatory changes that cause the phenotypic change is difficult, if not impossible (Stern and Orgozozo 2008).

Our understanding of how natural selection alters gene regulation to produce adaptations begins with the discovery of transcription factors that bind specific regions of DNA and alter enzymatic activity in *Escherichia coli* (Jacob and Monod 1961). Stemming from this discovery, it was hypothesized that phenotypic evolution is heavily influenced by *cis*-regulatory elements. This helped shift the field of evolutionary biology from one focused on coding-region mutations to genetic variation in gene regulation (Stern and Orgozozo 2008). *cis*-regulatory evolution appeared to be even more important at driving evolution in multicellular organisms since their complex development requires precise timing and differential expression of genes in order to

produce different cell types, tissues, and structures from a single genome (Ptashne and Gann 2002). With this new understanding, evolutionary developmental biologists have worked intensively to map gene regulatory networks (Stern and Orgozozo 2008). With a growing focus on the importance of gene regulation for adaptation, there has been some push back and arguments that the necessary experimental evidence has not been generated to merit some of the strong claims made (Hoekstra and Coyne 2007). In the end, this debate has been predominantly concerned with quantifying the contributions of coding versus *cis*-regulatory mutations to phenotypic evolution (Stern and Orgozozo 2008). However, this debate may have distracted from the primary goal of being able to predict evolution (Stern and Orgozozo 2008). To this end, the field requires studies that can precisely define how mutations cause phenotypic change in traits under selection. A part of this is to understand how mutations in gene regulation produce adaptive phenotypes.

There are several fundamental questions with regard to gene regulation evolution that remain relatively unanswered. For example, how can random mutations in gene regulatory elements produce adaptive phenotypic variation? How do genetic regulatory networks respond to evolutionary pressure? Some progress has been made through studies on viruses. These noncellular organisms have simplified genomes and gene regulation, which makes them an idea model system to study (Wittkopp, 2013, p. 413). Using a genetically modified HIV strain, researchers were able to evolve variants in the laboratory with gene regulation of the Tet-on system that maximized the efficiency of the system in response to doxycycline (Das et al. 2006). Studies on bacteriophage λ have shown that natural selection acts on gene regulation that determines when the virus lyses its host cell in order to optimize its reproduction (Wang 2006).

Mathematical models of this gene regulatory system have even shown that selection can favor stochastic gene expression (Singh and Dennehy 2014).

Given the previous work on cell lysis time gene regulation, we designed a series of evolution experiments with bacteriophage λ. λ infects *Escherichia coli* and is a valuable model organism because it has a small genome and short generation time, which makes it ideal for evolution experiments (Meyer et al. 2016). λ lysis timing is largely controlled by the S gene (Young et al. 1995). This one gene encodes two protein products, a holin protein that perforates the cell membrane, and an anti-holin protein that nullifies the action of its partner (Young et al. 1995). These products are produced at a 2:1 ratio until a threshold of approximately 4,600 active holin molecules is reached, and then the accumulated holin proteins trigger cell lysis (Young et al. 1995). This antagonistic system keeps λ from lysing the host early in the replication cycle to give viral progeny a chance to assemble (Young et al. 1995). With two different start codons, the translation of these two proteins is regulated by two RNA loops that provide access to the two separate Shine-Dalgarno sequences where translation initiates (Young et al. 1995). Extensive research has already been conducted on how point mutations and deletions in the regulatory areas of the S gene can alter the timing of lysis by altering the relative production rate of the two gene products (Young et al. 1995). Moreover, there is an established tradeoff between burst size and lysis time such that λ can extend lysis time to build more particles (7.7 per minute), at the cost of delaying the infection of new host cells (Wang 2006). This tradeoff means that λ experiences pressure to optimize lysis time to make the most of the *E. coli* cells in its environment, and that λ can be pressured to evolve shorter or longer lysis times by altering its supply of host cells.

We hypothesized that λ could be selected to evolve shorter lysis times by stopping λ infection just before the typical virus lyses its host cell. We also hypothesized that λ could be selected to lengthen its lysis time by stopping λ infection just before it has time to undergo two infection cycles. To test this, we evolved λ bacteriophages under long and short selection regimes and measured a number of characteristics of the evolved λ's genomes and phenotypes. We compared their growth dynamics to the ancestral virus in order to test whether our selection regimes selected for changes in lysis timing. We sequenced the S gene to determine whether mutations in this locus caused changes in lysis time. We also engineered a naïve genome with an *S* mutation in order to isolate the phenotypic effects of the mutation. We measured the burst size of the virus to test for a trade-off between lysis timing and particle production. And lastly, we measured the fitness of the evolved λs in order to determine how lysis regulatory changes drive adaptive evolution.

Materials and Methods

Phage and Bacterial strains

The ancestral phage λ strain used in our study was a mutant of cI857 (provided by Ing-Nang Wang, State University of New York at Albany) that had been engineered and preadapted by former lab member Everardo Hegewisch to have three mutations in the *J* gene (GA18823, CT18503, AG18535) that increased the rate of binding to host cells. We used this strain to reduce the possibility of our evolution protocol selecting for phages that infect faster instead of phages that can lyse their host faster. Phage stocks were created by combining 4 mL of LBM9 (20 g tryptone, 10 g yeast extract, 12.8 g sodium phosphate heptahydrate, 3 g potassium phosphate monobasic, 0.5 g sodium chloride, 1 g ammonium chloride, 20 mM magnesium

sulfate, 22.2 mg calcium chloride per L water) in a tube with 100 µl of *Escherichia coli* (WT, K-12 BW25113) (Baba et al. 2006) inoculated with λ from a freezer stock, and cultured overnight in a 37°C incubator shaking at 220 rpm. The next day stocks were taken out of the incubator and transferred to 15 mL falcon tubes, then 100 µl of chloroform added and centrifuged at 15,000 rpm for 10 minutes. The remaining supernatant was removed, placed in a sterile 15 mL falcon tube, 75 µl of chloroform was added, and the culture was stored at 4°C. For each culture, a freezer stock was made removing 1ml of the phage culture into a freezer tube with 200 µl of 80% glycerol. These were the standard protocols for culturing and preserving our phage stocks. *E. coli* strains were stored frozen at -80°C with 15% v/v glycerol, and only 100% wildtype cells were used for this project. Bacterial strains were cultured from these frozen stocks in sterile glass tubes and adding 4 mL of LB (Lennox Broth: 10 g tryptone, 5 g yeast extract, and 5 g sodium chloride per liter water), then incubated overnight at 37°C incubator shaking at 220rpm.

Lysis Time Determination

Before we initiated the evolution experiments, we quantified cI857's normal lysis time to determine when we should arrest growth to select for short or long lysis times. The short protocol would be set just before the first burst of the cells to select for fast lysis, and then just before two times the lysis time in order to select for slower lysing λs. Though it seems stopping growth before initials bursts would kill all of our phages, there is enough variance in lysis timing that many of the phages would survive to propagate in the next cycle. We also used this protocol to compare the phenotypic effect the first 10 days of evolution had on the long and short evolved λ to their ancestor. We combined 9.8 mL of modified LBM9, 100µl of wildtype *E. coli*, and 10 µl of our CI857 3 J mutant λ phage stock made from an overnight infection culture in a

50 mL Erlenmeyer flask. 5×10^8 cells and ~10^5 λ particles were used to initiate each population. Three cultures were made and placed in the 37°C shaking incubator for four hours at 120 rpm. Every twenty minutes, the flasks were taken out, and a 300 µl aliquot was removed, the flasks went back in the incubator, and 50 µl of chloroform was added to each aliquot to kill bacteria and thus stop phage growth. between 80 min and 120 min aliquots were removed every ten minutes to increase the resolution around the expected time of lysis for our phage. After sampling for four hours, we diluted each aliquot and spotted 2 µl of each series on large LB Lennox plates with 1 mL WT *E. coli* and 10 mL of soft agar (10 g tryptone, 1 g yeast extract, 8 g sodium chloride, 7 g agar, 0.1 g glucose per liter water, supplemented with a final concentration of 2 mM calcium chloride and 10mM magnesium sulfate), and then placed in the 37°C incubator overnight. The next day plaque were counted from the spot plates and converted into pfu/mL. As an informal analysis to understand if our evolved phages are bursting earlier than the ancestor, for each timepoint the current and previous density measurements were used to calculate the percentage of how much the density decreased or increased between the two time points. This, in turn, allowed measurable differences between our evolved strains of λ in how they grew and changed over time. For a single timepoint percent change was calculated as [((*density of timepoint / density of previous timepoint*) - 1) * 100].

Evolution Experiment

The protocol is outlined in Fig. 1A. Eight populations of a lysogenic strain of λ (cI857 3J) that was induced into a lytic phage were cultured for 115 min in a 37°C shaking incubator at 120 rpm. Each population was grown in a flask using the same requirements from the lysis time determination protocol, except only 9.7mL of LBM9 was used, and 200 µl of WT *E. coli* added.

At 115 min the flasks were taken out of the incubator, 1.5mL aliquots were removed and centrifuged at 15,000 rpm for one minute. The supernatants of four populations that were subjected to the short evolution protocol were removed and saved for the next round of evolution, and the pellet was discarded. The supernatant of the other four flasks in the long protocol was removed, and the pellet was resuspended in 1.5 mL of LBM9 and placed in a 37°C water bath for 30 minutes. During this time another 1.5 mL aliquot was removed from each short and long flask and mixed with 300 µl of 80% glycerol in a 96-deep well plate and placed in the -70°C freezer. After 30 minutes the four long populations were centrifuged again at 15,000 rpm, then the supernatant was removed and saved for the next day of evolution. All aliquots were stored at 4°C until the next day. This protocol was repeated for 10 days, except each following day 1 mL of the aliquot was deposited into their corresponding flask, and the volume of LBM9 was adjusted to keep the total volume at 10 mL.

Mutation Identification

We sequenced the λ *S* gene from our evolved cI857 3J phages using traditional Sanger sequencing techniques (see Table 1 for primer sequences), and automated ABI sequencers operated by Genewiz (La Jolla, CA).

Mutation Engineering

We found only one mutation from one evolved λ population (L3.1) from the long protocol. We introduced the mutation into a lysogen, cI857. Modifications were made using Multiplexed Automated Genome Engineering (MAGE) following identical protocols used in

Meyer *et al*. 2016 (Isaacs et al. 2014). See Supplementary Table 2 for oligo sequences used for transformation and sanger sequencing verification.

Mutational Effect on Phenotype

L3.1 had a mutation in the *S* gene that we predicted would alter the normal phenotype, but it was important to confirm that our mutation caused the change in *S* gene expression and not a side effect of our evolution protocol. To test this, we triggered cell lysis by heat shocking lysogens. High temperatures denature the λ cI repressor protein in cI857, signaling *S* expression and lysis to begin. This gives us accurate readings of lysis timing since nearly all cells in the population are triggered simultaneously to lyse. This highlights the importance of studying a lysogenic strain of λ since in a lytic strain lysis time cannot be as easily measured because it would have to undergo several other infection processes such as virion assembly and genome replication that introduce heterogeneity in timing. Lysogen cell densities (OD) were observed for four hours after the heat shock. If the *S* mutation extends lysis, then the cI857 lysogen modified with the S mutation will hold a higher OD for longer compared to the unmodified lysogen, or if lysis is shortened then OD would start to drop earlier. Heat shock was performed, according to Meyer *et al*. 2016. After heating the culture, a 200 µl aliquot was removed and placed in a Costar flat-bottom 96-well microtiter plate. Four samples from cI857 and four from the *S* mutant (L3.1) were heat-shocked and analyzed, while four samples from both cI857 and *S* mutant were left at room temperature to act as a heat-shock control. For four hours the OD of each well was recorded every minute using a Tecan Sunrise with an Absorbance 600nm, Infinite

F200 PRO/F500, NIP filter that was set to 37°C and shook for five seconds before each measurement.

Competition Assay

To compare the fitness of the evolved strains relative to their ancestor in the long protocol where the mutation evolved, we directed competition experiments between 3 different λ (a long-evolved phage without the mutation, a genetically modified phage with our S mutation, and a short evolved phage) against a genetically identifiable version of λ that creates blue plaques. This phage was created by recombining the *alpha* region of the *lacZ* gene in *E. coli* into the *R* gene of λ (cI26) (Burmeister et al. 2016, Shao and Wang 2008). When X-gal (bromo-4-chloro-3-indolyl-β-D-galactopyranoside) is added to the agar it reacts with LacZ to form blue plaques on an agar plate that can be distinguished from normal clear plaques.

To ensure that blue coloration was due to marked phage and not *E. coli*, phages were plated on DH5α which has *lacZ*α deleted. Before cooling, 0.5 mg per ml of X-gal, and 0.25mg per ml Isopropyl β-D-1-thiogalactopyranoside was added to the soft agar. Blue and clear plaques were counted on each plate, converted to densities, and per cycle relative fitness was calculated as:

[*ln*(*final density of evolved λ/initial of evolved λ*)]-[*ln*(*final density of blue λ/initial density of blue λ*)] (Travisano and Lenski 1994).

Burst Size Analysis

To measure the burst size of our phage with the *S* gene mutation, we experimentally determined the burst size of our mutated λ, the ancestor, and another λ that had evolved in the long protocol but did not acquire the mutation. Theoretically, if the *S* gene mutation extended lysis time, then the burst size will be bigger compared to the ancestor. To start, we mixed together 2-3x10^7 pfu/mL of phage with 2x10^8 cells/mL of WT and 5mM $MgSO_4$ in a flask to a final volume of 10mL in LBM9, then incubated in the 37°C shaker to grow for 15min at 120 rpm. Once complete, each culture was diluted 10^4-fold in tubes of prewarmed LB broth at 37°C to a final culture volume of 10mL to be able to have countable plaques on a soft agar plate. Afterward, we removed 1 mL aliquots from the final dilution to use for infusion plates with 100µl of WT cells and 4mL of agar to get the total concentration of phage (*T*). We removed another 1ml aliquot from the culture, added 20 µl of chloroform and vortexed, then plated to get the concentration of unabsorbed phage (*U*). Then the remaining culture was placed in the shaking incubator at 37°C for 75 min at 220 rpm, added 20 µl of chloroform, shook for five more minutes in the incubator, then diluted and plated to get a final concentration of phage (*F*). Burst size was calculated using this formula: $b=F/(T-U)$ (Wang 2006).

Results and Discussion

EVOLUTION PROTOCOL COMPARISON

For the short-evolved λ we expected to see an earlier increase in density before the ancestor, and the long-evolved λ densities would rise later than the ancestor. The short λ had a notable decrease in lysis timing with two distinct large jumps in density at 50 and 120 minutes

over 160 minutes, whereas the ancestor achieves only one round of infection (Fig. 2a). Bursting ten minutes before the ancestor at 60 minutes, λ shortened its infection cycle by 16.67 %, which is a similar effect to an *S* gene mutant that was missing its Shine-Dalgarno sequence for S107 and shortened λ's infection cycle by ~15% (Young et al. 1995). This result shows that wildtype λ has a slower infection cycle than what can be achieved, and that it can easily be selected to hasten its infection cycle. What this means for viruses more generally is that they likely experience selection in nature to optimize the timing of their infection cycle, rather than to just infect as fast as possible. This is in line with previous observations that λ under evolutionary pressure will be directed towards intermediate lysis times, where it has the largest gain in fitness (Wang 2006).

Surprisingly, the long λ emulated this effect, defying the expectations of our evolution protocol (Fig. 1a). Calculating the change (%) in phage density between two consecutive time points (Fig.2b) showed the short λ had a larger significant increase at 50 minutes (T-test, $p = 0.0022$) as compared to the ancestor, while the long λ also had a large increase in density (relative to the ancestor) at 50 minutes that was nearly significant (T-test, $p = 0.0506$) (Fig. 2c). At 60 minutes the ancestor had a large increase in phage density, and both long and short λ have significantly lower density shifts (T-test, $p = 0.031$ and $p = 0.031$) during that time compared to the ancestor (Fig 2d). Based on these results, the short λ had evolved to burst 10 minutes earlier than the ancestor, matching our predictions that it would evolve faster lysis times. The long-evolved λ resembled the short λ because it also had a large increase in density at 50 minutes. This was confusing because we predicted that our long evolved λ would maximize its fitness by evolving the longest lysis time possible to increase the number of progenies it produced. What took us by surprise was that if λ could reach a lyse a second round of infection before the phage

was harvested, it could be fast enough to achieve a large increase in fitness. For example, each extra minute a phage stays within the cell, it will produce 7.7 more particles, and on average our wildtype phages take about 125 minutes to infect and lyse the cells (Wang 2006). Our long protocol gave the phages an extra 10 minutes to lyse, and if they evolved to use that time, they could produce an extra 77 particles per infection. However, the phages evolved to speed up their infections by about 10 minutes, while making less progeny per cell, they can undergo two rounds of infection and yield more particles. Normal cI857 makes 153 particles per cell, so the evolved lambda will only make 76 particles per cell, but each of these particles will go on to produce 76 more particles. Therefore, a wildtype λ will make an average of 154 particles in a single round, whereas the evolved one will produce 76 times 76 (5,776). This amounts to a sizeable fitness increase and explains why there was an enormous reward for faster-lysing phages even in the long protocol.

Following this logic, it is surprising that selection favored a mutation that extended lysis time instead of shortening it. While it did delay mean lysis time, it extended the tails of the distribution of lysis time, which may have had a significant adaptive effect. By stretching the tails of the distribution, more λ were able to lyse their hosts before the wildtype. This, in turn, allowed early-bursting λs a chance at re-infecting a host and amplifying its progeny production to receive the huge fitness gain outlined in the previous paragraph. Because of this major benefit derived from reaching a second infection cycle in our protocol, it did not matter if the S mutation increases lysis time. The bottom line may be that selection does not care about how the mean of the population shifts, but the number of particles that lyse before the wildtype, which can be achieved through either shifting the mean or enhancing stochasticity in lysis time.

λ MUTATION RESPONSIBLE FOR ADAPTATION

We predicted that λ would evolve mutations in the *S* gene since the anti-holin and holin proteins produced by it are responsible for governing lysis time. Sanger sequencing of this gene (~400bp) and about 100bp and up and downstream revealed no changes in the S gene for any short or long evolved λ except for one, the first indication that our evolution protocol was not selecting for *S* gene expression like it was designed to. Alternatively, our protocol could have selected for phages that infect their host faster instead of lysis, even though we tried to counteract that possibility by using phage that were pre-engineered to be better at infection. Another possibility is that we put selection pressure on genes that control virion assembly and increasing the rate that progenies are created, or a gene duplication that doubled the rate of S105 production would have lowered the time needed to lyse their host.

The one λ to receive a mutation (L3.1), had a mutation in the transcribed region of the *S* gene and the 5' UTR of its mRNA. A 'G' nucleotide was inserted in both the Shine-Dalgarno sequence for the anti-holin protein (S107) and outside the base of the upstream regulatory mRNA stem-loop that influences the translation of S107 (Fig. 4). This mutation could affect translation of S107 by de-stabilizing the upstream RNA loop which provides ribosomes access to the start codon for anti-holin. With increased ribosomal access to the Shine-Dalgarno sequence, then translation of S107 would be upregulated. However, m-fold determined the mRNA strand with the mutation is marginally more stable with a ΔG of -20.28 compared to a ΔG of -19.38 when the mutation is taken out, while maintaining the same folding structure as the wildtype mRNA strand (Fig. 4). It is difficult to determine the effect this insertion will have on translation just by its location alone, however, by somehow increasing the rate of translation of S107, it

would disrupt the normal ratio at which both S107 and S105 are produced, meaning the time to reach the threshold needed to stimulate lysis would take longer than normal.

EFFECT OF MUTATION ON LYSIS TIMING

To reconcile the divergence between the observed phenotype and the predicted effect on S gene regulation, we tested the effect this mutation has on cell lysis timing. To do this, we engineered the S mutation using MAGE into an unevolved strain of λ (cI857), which created a lysogen that only had our insertion and no mutations potentially present in the un-sequenced portion of our evolved genomes. After stimulating lysis in our engineered λ and an un-engineered cI857 control we recorded the optical densities of the cultures over four hours to determine when cell lysis was triggered. Cell lysis happened later with the S mutation, but the OD had a much wider peak meaning a wider range of expression of the *S* gene (Fig. 5a). This indicates a lower lysis rate over time until the peak where host death rate overtakes growth but at a slower rate than the ancestor. Analysis of the peak times indicated a significant increase in the time before OD started decreasing, meaning the mutation overall increased lysis time, confirming our predictions from the placement of the insertion mutation (Fig. 5c, T-test, p = 5.2e-9). However, the data also seems to indicate an increase in stochasticity of gene expression, with some λ also lysing earlier than the ancestor as well. This was confirmed by normalizing the OD of each the heat-shocked λ with their room temperature counterparts. By subtracting the OD value of the heat-shocked from the room temperature OD, it outlines a higher rate of cell death that balances out the growth just after stimulation of lysis. This meant a minority of λ were lysing their hosts much earlier than the ancestor as well (Fig. 5b). This mutation was likely

adaptive because it allowed λ to produce a few very fast lysing phages that could spread to new cells, while paying the cost of producing a majority of slow lysing kin.

DARWINIAN FITNESS

Competition experiments confirmed that our evolution protocol resulted in large fitness increases for λ, but not necessarily due to changes in *S* gene expression as we expected. The short-evolved λ had large increases of fitness in the long protocol, which demonstrated that evolving through our protocol conferred adaptive changes with correlated benefits in multiple environments and that even the so-called long protocol favored short lysis times (Fig. 3). Moreover, the long evolved λ without the mutation almost matched the fitness gains of the short evolved λ (Fig. 3). When we tested the fitness effects of just the S mutation on λ, there was only a minor increase in fitness (Fig. 5d) that only accounted for part of the increase in fitness of the evolved λs, suggesting that adaptation occurred in areas other than the *S* gene.

BURST SIZE TRADEOFF

Since the S mutation was confirmed to increase lysis time, we tested whether the burst size also increased according to the lysis time / burst size tradeoff previously reported (Wang 2006). While overall the average burst size had increased in λ with the mutation alone, the increase was not significantly different from the ancestor (Fig. 6, T-Test, $p = 0.19$). Additionally, a different long λ isolate without our mutation had a burst size similar to the ancestor and had a lower average burst size compared to λ with the mutation, which indicates that burst size may be increased by our *S* mutation (Fig. 6).

Conclusion

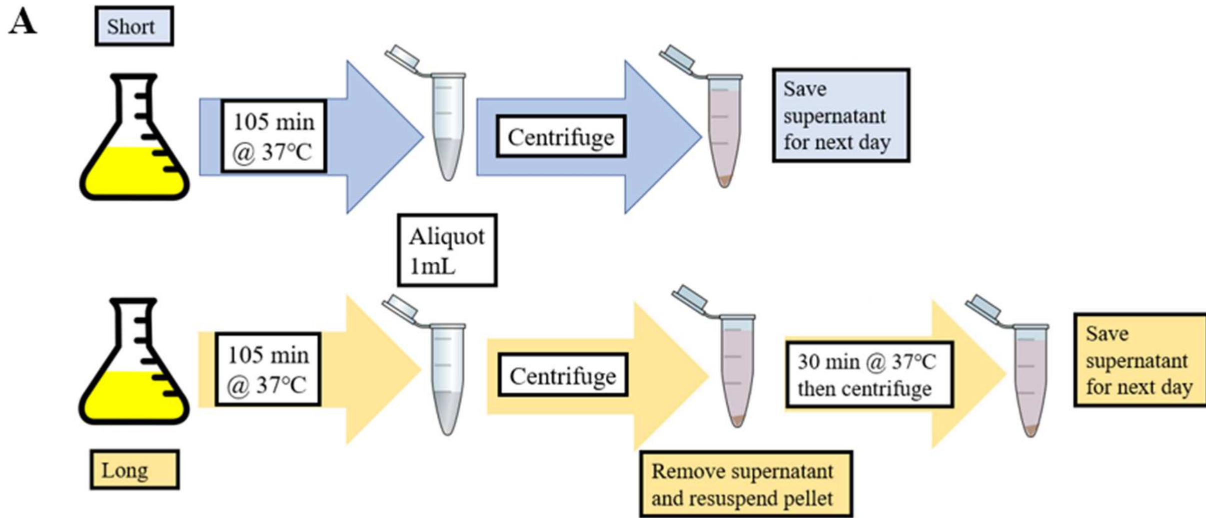
Two of our most confounding results were the facts that fast-lysing phages evolved in treatments that should have selected for delayed lysis, and the lack of genetic changes to the *S* gene. While reviewing the literature, we found a similar study on bacteriophage T7 that discovered similar unintuitive responses to lysis timing selection. These evolution experiments yielded unexpected responses in timing and adaptation did not occur in a gene homologous to *S* (Heineman and Bull 2007). Researchers found that T7 evolved towards having earlier lysis times when pushed to lyse more quickly, but it did not evolve a delayed lysis in an environment that favored late lysis. The study also found little genetic evidence to support the changes in lysis timing (Heineman and Bull 2007). Their explanation for the disconnect between their expectation and the path T7 evolution took was that it might have uncovered mutations in a trait that allowed it to break the lysis-burst size tradeoff (Heineman and Bull 2007). This explanation does not appear to explain λ's evolution since the variants we measured burst sizes for seem to fall along the tradeoff. Both studies demonstrate the need to develop more advanced understandings of gene regulatory evolution in order to successfully predict evolution.

One of the most compelling results we uncovered was an *S* mutation that increased the stochasticity of lysis timing and thus the expression of holin and antiholin yet was adaptive. While at some level, all gene expression is stochastic (Paulsson 2005), high levels of stochasticity are typically thought to be deleterious since there is normally a single phenotype best adapted to a given condition, so producing random variation among progeny will necessarily yield some unfit individuals. Although, under some scenarios, gene expression stochasticity can provide advantages as well (Kaern et al. 2005). Higher variability leads to greater plasticity, which leads to an elevated probability that no matter how the environment changes at least some

individuals may persist (Macneil and Walhout 2011). For example, it can give cells the plasticity needed to react to sudden stresses and acclimate to fluctuating environments, and act as a means for populations to form heterogeneity during cellular growth and differentiation (Kaern et al. 2005). Gene regulation stochasticity is commonly observed in viruses, however; it has been observed in organisms ranging from microbes to eukaryotes, and it can depend on the genetic regulatory framework and the biophysical constraints that manage gene expression (Raj and Oudenaarden 2008). λ in our study was exposed to a stable environment, so gene expression stochasticity as an adaptive route to cope with variable environments was unlikely. Rather, we predict that this mutation was a short-term solution to overcome the obstacles presented by selection, and that in the long-run, *S* mutations will arise that fine tune its gene expression and cause less variability in lysis timing. This prediction stems from a study that showed stochastic S gene expression in λ reduces its fitness under stable conditions (Singh and Dennehy 2014).

Phenotypic stochasticity seems to be a prominent feature of viruses and is thought to be due to that fact that their survival depends on biological processes such as apoptosis, latency reversion, and lysis stimulation to complete replication cycles that are subject to randomness because they are stimulated by specific concentrations of effector molecules that can fluctuate based on subtle changes in gene expression (Singh and Dennehey 2014). Another example of gene stochasticity in viruses includes herpes virus latency, where the fittest variant in fluctuating environments is the one that produces progeny with the most variation in latency expression (Stumpf et al. 2002). While stochasticity could be a simple consequence of being a virus, our experiment reveals that it can be used as a phenotypic heterogeneity generating mechanism that helps produce adaptive variation.

FIGURES



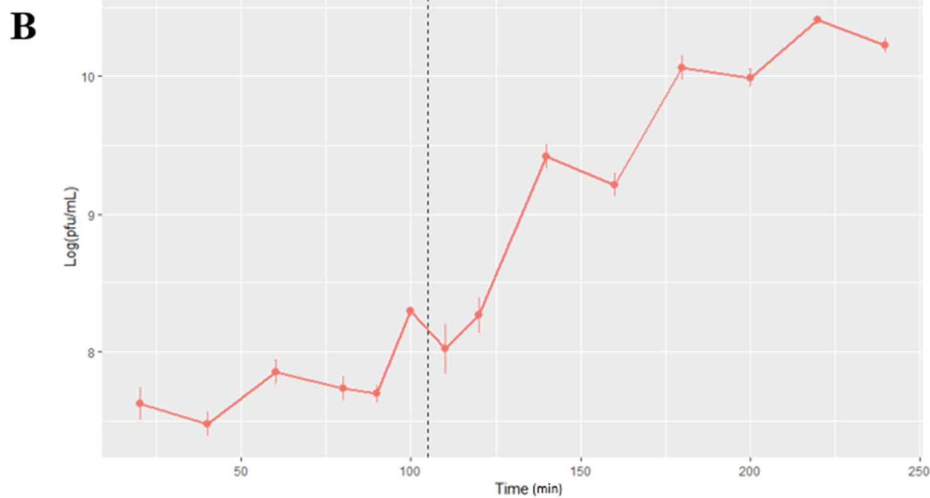


Figure 1 **Evolution Protocol & Lysis Time Determination**

(A) The illustration represents the evolution protocol and steps required to select for shorter or longer lysing phages. **(B)** Standard growth curve of our ancestral cI857 λ to determine normal lysis time before undergoing evolution to understand where to stop growth before lysis. The dotted line indicates the time when growth was stopped for λ before selecting for phages. Error bars denote 95% confidence intervals.

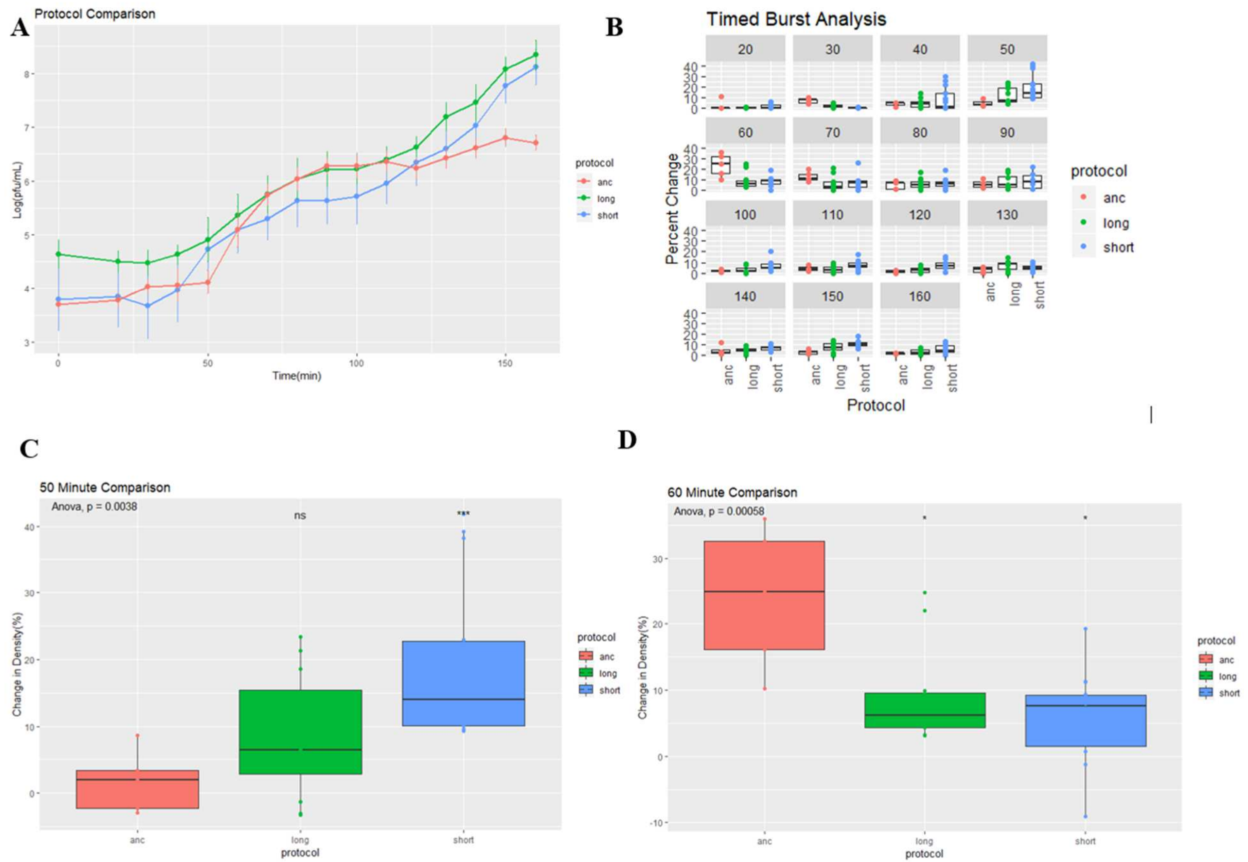


Figure 2 **Protocol Comparison & Informal Burst Analysis**

(A) Growth curves of the newly evolved short and long phages compared to the ancestor. The blue line is short, the green line is long, and the red line is the ancestor. Error bars denote 95% confidence intervals. **(B)** Analysis of the growth curve data to estimate the change in λ density between two consecutive time measurements. Error bars indicate 95% confidence intervals. **(C)** A closer examination of the 50-minute timepoint and comparing the density changes between all protocols. Error bars denote 95% confidence intervals. See text for stats. **(D)** Closer examination at the 60-minute timepoint and comparing the density changes between all protocols. Error bars denote 95% confidence intervals. See text for stats.

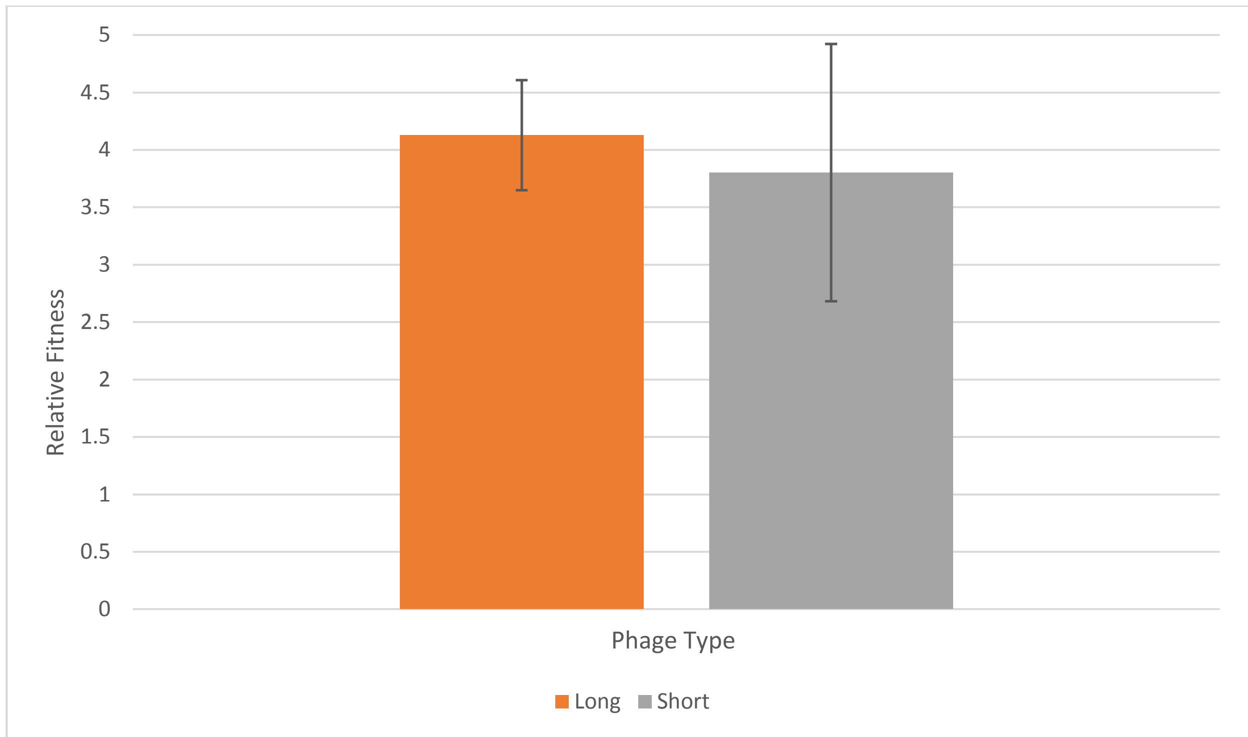


Figure 3 **Protocol Competition Assay**

Competition assay comparing how differently evolved phage handle surviving in the long evolution protocol. Values significantly above 0 indicate fitness gains. Error bars denote 95% confidence intervals.

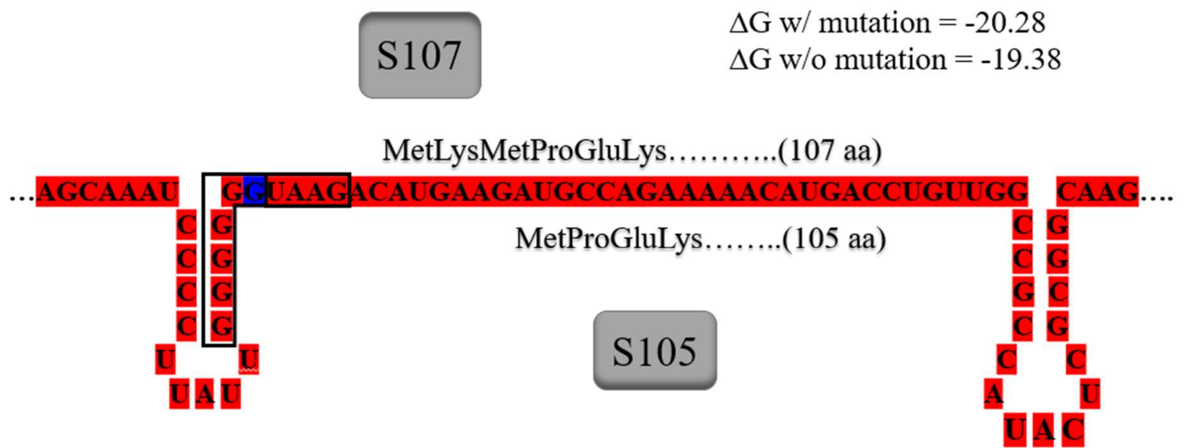


Figure 4 **Insertion Mutation**

An illustration of the several regulatory regions in the mRNA of the *S* gene as well as the location of the discovered insertion mutation highlighted in blue. There are two different start codons, one for the anti-holin (S107) and holin (S105) proteins, an upstream and downstream stem loop that regulate the production of the S107 and S105 proteins respectively, and two Shine-Dalgarno sequences boxed in black for initiation of translation for S107 and S105 respectively. RNA folding form predictions give two different free energies for the two mRNA strands.

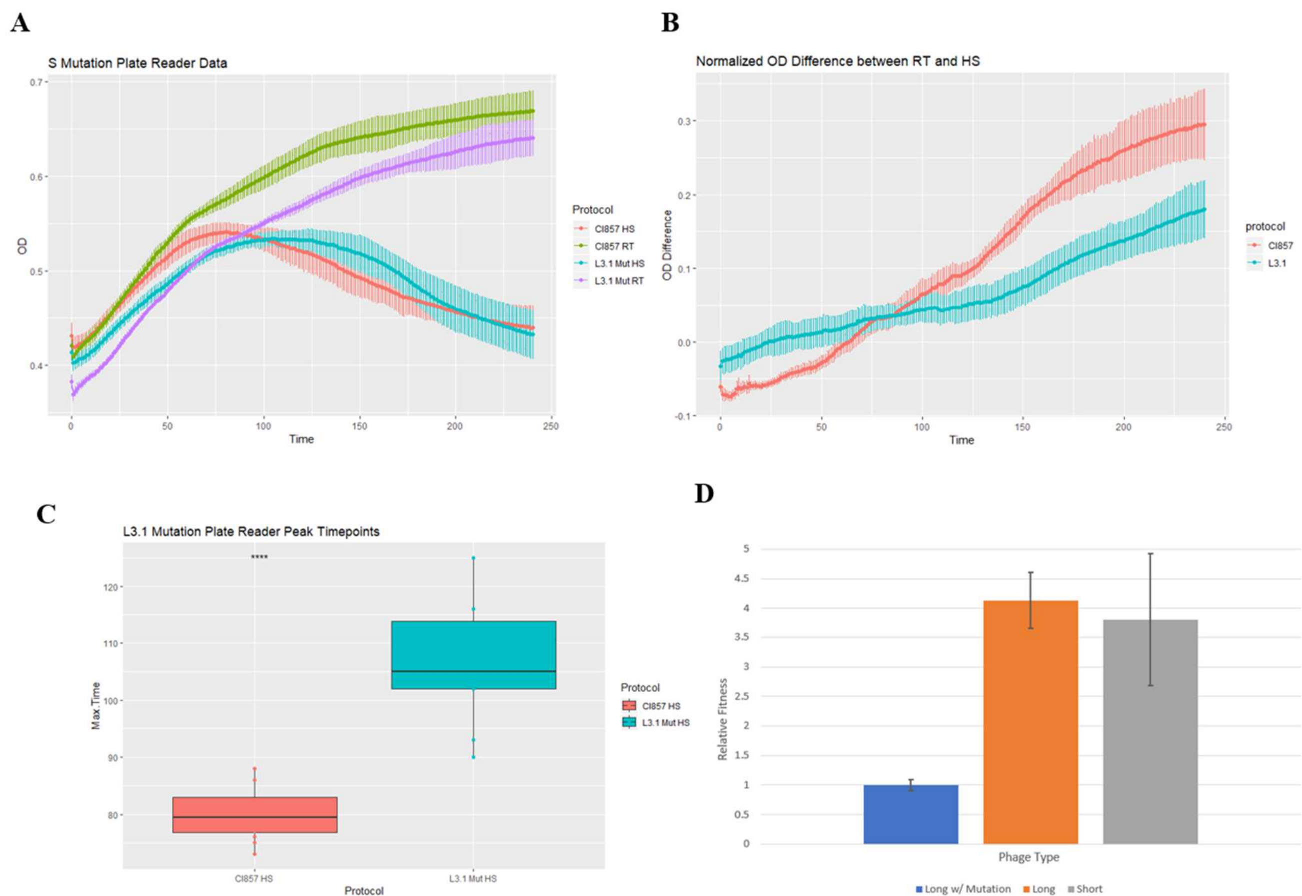


Figure 5 **Mutational Effect on Lysis Time & Fitness Assay**

(A) Optical density measurements to quantifying cell lysis from λ induction with mutation in blue, and ancestor in red. Error bars show 95% confidence intervals. **(B)** Normalized height difference in optical densities between the room temperature and heat shocked phages. Error bars show 95% confidence intervals. **(C)** Comparing the time at which cell death reaches its peak and starts to decline. Error bars show 95% confidence intervals. See text for stats. **(D)** Competition assay comparing differently evolved phages fitness to just the mutation in the long protocol. Error bars show 95% confidence intervals.

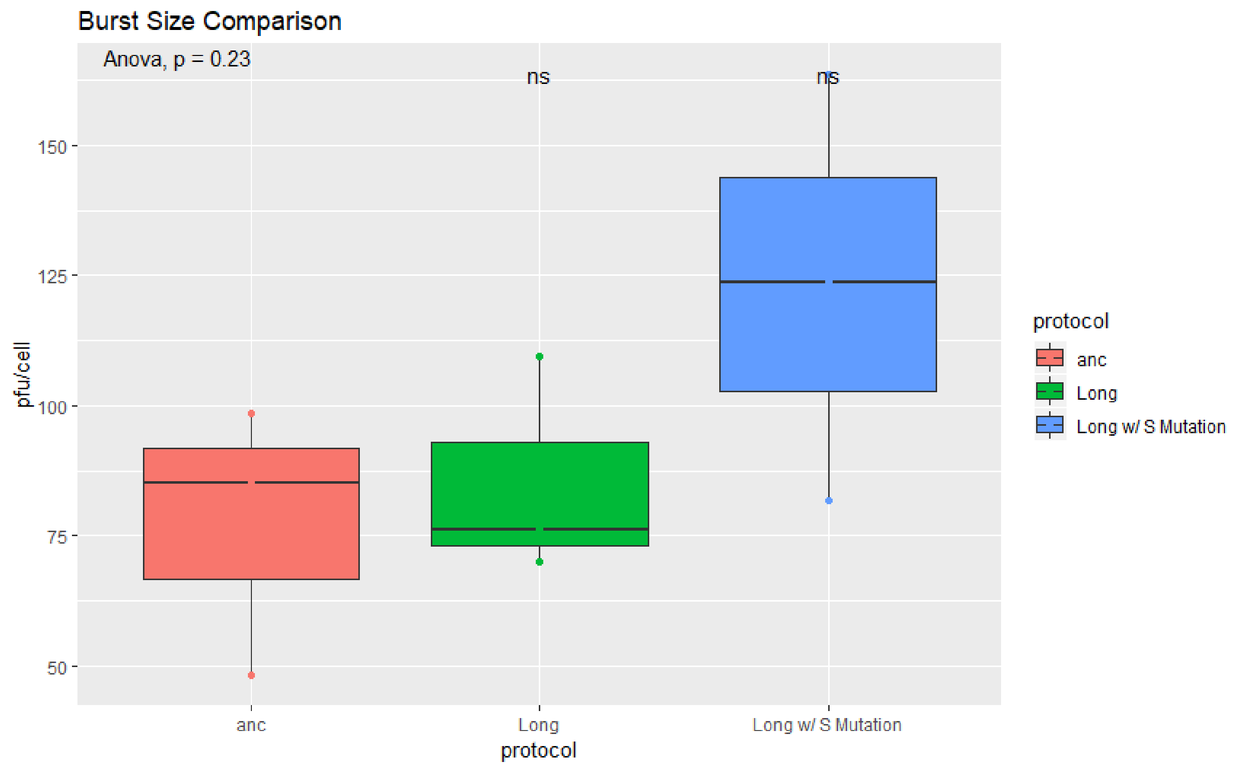


Figure 6 **Burst Size Comparison**

Burst size of λ from its host for a phage with our mutation in blue, a long-evolved phage without our mutation, and their ancestor in red. Error bars denote 95% confidence intervals. See text for stats.

TABLES

Table 1 **S gene primer sequences**

NAME	SEQUENCE (5’-3’)	USE
SF1	GCACAACAGGTAAGAGCATTG	Amplification and Sequencing of *S*
SF2	CCCACCAACGGGAAAGAA	Amplification and Sequencing of *S*

Table 2 **Oligos used for genetic engineering**

Asterisks indicate phophorthioated bonds and lowercase letters indicate the bases directing mutations in the oligo.

Name	Mutations Introduced	Sequence (5’-3’)
S_mut_oligo	G318	A*A*T*G*GCGGCCAACAGGTCATG TTTTTCTGGCATCTTCATGTCTTACC CCCCAATAAGGGGATTTGCTCTATT TAATTAGGAATAAGGTCG

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