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LETTERS

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Natural selection underlies apparent stress-induced mutagenesis in a bacteriophage infection model

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The emergence of mutations following growth-limiting conditions underlies bacterial drug resistance, viral escape from the immune system and fundamental evolution-driven events. Intriguingly, whether mutations are induced by growth limitation conditions or are randomly generated during growth and then selected by growth limitation conditions remains an open question[1]. Here, we show that bacteriophage T7 undergoes apparent stress-induced mutagenesis when selected for improved recognition of its host's receptor. In our unique experimental set-up, the growth limitation condition is physically and temporally separated from mutagenesis: growth limitation occurs while phage DNA is outside the host, and spontaneous mutations occur during phage DNA replication inside the host. We show that the selected beneficial mutations are not pre-existing and that the initial slow phage growth is enabled by the phage particle's low-efficiency DNA injection into the host. Thus, the phage particle allows phage populations to initially extend their host range without mutagenesis by virtue of residual recognition of the host receptor. Mutations appear during non-selective intracellular replication, and the frequency of mutant phages increases by natural selection acting on free phages, which are not capable of mutagenesis.

Until the late 1980s, following classical experiments by Luria and Delbruck, Newcombe and the Lederbergs[2–4], it was widely accepted that mutations arise as random pre-existing events that are selected by growth limitation conditions, rather than as induced events occurring in response to such conditions. Those experiments temporarily ended the long Darwinism–Lamarckism debate that had begun a century earlier.

Approximately 25 years ago, however, experiments with *Escherichia coli* selected under growth-limiting but non-lethal conditions demonstrated that mutations accumulate and appear to be induced by stress in a non-growing cell population[5]. This population carried a frameshift mutation in *lacZ*, a gene essential for lactose utilization. On medium containing lactose, these non-growing cells appeared to reverse their *lac* mutation at a higher frequency than that expected to occur during normal growth[6]. The reversion mutation did not pre-exist, but rather appeared after nutrient deprivation. Furthermore, fluctuation tests carried out under these conditions showed a Poisson distribution of the number of mutants, as opposed to the stochastic fluctuating number of mutants obtained by Luria and Delbruck in their experiments, suggesting that the revertants arose after plating on selective medium[5].

Proponents of the 'stress-induced mutagenesis' (SIM) model proposed that the higher mutation rate occurs in response to the growth limitation[5,7,8], whereas opponents explained the rates in terms of slow DNA replication/cell division (hereafter termed 'cryptic growth') shaped by natural selection[9–13]. The renewed debate centred around whether the stressed cells induce mechanisms to cause increased DNA replication in the absence of growth, resulting in increased mutation rates, or whether cryptic growth occurs, allowing natural selection of randomly generated beneficial mutations.

Several biological systems were presented, but none decisively demonstrated lack of cryptic growth during the encountered growth limitation[7,9–11,14]. It was suggested that the major reason for the 150 years of irresolvable conflict over the origin of mutations is the lack of a simple model in which the effects of selection and mutation are separated[15].

Here, we have designed a simple experimental system in which the effects of selection and mutation are separated, to demonstrate that growth limitation selects for beneficial mutations but does not induce them. The apparent SIM, characterized by higher than expected mutations, occurs in a bacteriophage-based model in which DNA replication is physically and temporally separated from the growth limitation. DNA replication, during which mutations may accumulate in the phage, occurs exclusively inside the host. The encountered growth limitation, that is, phage receptor recognition, takes place exclusively outside the host. Our results provide decisive evidence for natural selection occurring under growth limitation but not being induced by it.

We previously isolated *E. coli* mutants that restrict bacteriophage T7 infection due to incomplete lipopolysaccharide (LPS) production[16]. LPS is the recognition receptor for T7 bacteriophage, and a lack of genes encoding the enzymes required for LPS biosynthesis, such as *waaG*, results in host resistance to T7 phage, as the latter cannot adsorb properly to the bacteria[16,17] (Fig. 1a and Supplementary Fig. 1).

Nevertheless, some phages emerged on the *waaG* mutant *E. coli* (restrictive strain) at frequencies that were significantly higher than expected from the normal phage mutagenesis rate. These frequencies were measured by comparing the efficiency of plating (EOP) of T7 phage on the restrictive *waaG E. coli* host compared to an isogenic *E. coli* strain, referred to hereafter as the wild-type (WT) *E. coli* host. WT *E. coli* and *E. coli* lacking *waaG* were each mixed with T7 phages in soft agar and overlaid on LB–agar plates. Plaques were counted every 7 min during incubation at 37 °C. The EOP of the T7 phage on hosts lacking *waaG* following 160 min incubation was 0.032 ± 0.008 times that on WT hosts (Fig. 2). Thus, lack of the LPS receptor on the *waaG* host significantly reduced infection. However, the adsorption deficiency did not reduce the plaque formation capability of phages that entered into the cells, as infected cells, that is, centres of infection, plated with similar efficiency on *E. coli* lacking *waaG* compared to the WT *E. coli* (Supplementary Fig. 2). Following extended incubation (~370 min) of the phages under this growth limitation, the EOP increased to a maximum of 0.127 ± 0.002, indicating that new plaques had appeared on the *waaG* host. During this time, no additional plaques appeared on

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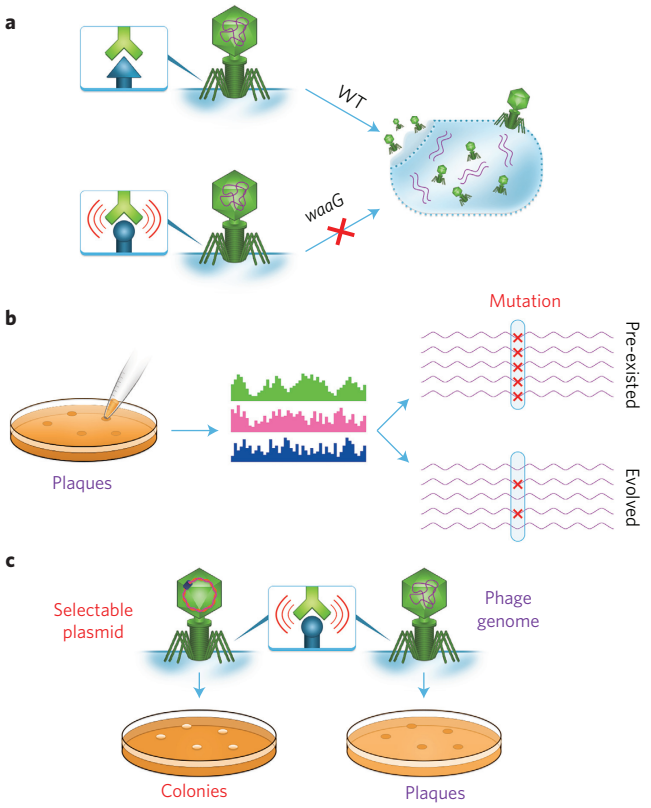

Figure 1 | Schematics of the experiments. a, T7 phage normally infects and propagates in WT *E. coli* hosts. On *waaG E. coli* hosts, the efficiency of plating is reduced due to the growth limitation of being unable to recognize the altered host receptor[16], but not due to DNA replication, which proceeds normally. **b**, High-throughput sequencing of a single plaque determines whether a mutation is pre-existing or evolved during plaque formation. Because plaques evolve from a single unit, a pre-existing mutation is expected to be identified in all progeny, whereas an evolved mutation will appear in only a fraction of the progeny. **c**, The ability of the phage particle to deliver DNA into hosts reveals the mechanism of cryptic growth. Phage particles injecting selectable DNA markers give rise to colonies, whereas particles injecting phage genomes give rise to plaques. Similar numbers of colonies and plaques obtained by these two different procedures indicate that the DNA injection is similar, regardless of the replication/mutagenesis ability of the plaques.

the WT *E. coli* hosts infected under the same conditions, indicating that a period of 160 min is sufficient for plaque formation.

The frequency (12.7%) of phages overcoming growth restriction appeared as SIM, as it was much higher than that expected from a random mutagenesis rate of $\sim 2 \times 10^{-8}$ to 7×10^{-7} mutations per base pair per generation measured for several phages[18]. For example, a mis-sense, non-sense or deletion mutation in almost any of the essential genes of T7 reduces the EOP to less than 1 in 10^6 (for example, refs 19 and 20).

To determine whether mutagenesis indeed occurred, we propagated the emerging plaques twice on the restrictive strain and then isolated single plaques. The resulting phages were DNA-sequenced in genes 12 and 17 (Supplementary Table 1, phages 2–3), or at the 3′ end of gene 17 (Supplementary Table 1, phages 4–13). Most phages (9 out of 12) carried mutations near the 3′ end of gene 17 and/or in gene 12, encoding the tail fibre and tail proteins constituting the T7-recognition ligand of the host receptor. Taken together, these results showed that the isolated phages accumulate mutations that overcome the growth limitation.

We further analysed high-throughput DNA sequencing of ten phages isolated after three growth cycles on either the *E. coli waaG* host or the WT *E. coli* host. These analyses indicated that the plaques isolated on the *E. coli waaG* host indeed had numerous mutations in genes 11, 12 and 17, encoding the tail and tail fibre proteins, including those identified in Supplementary Table 1, as expected (Supplementary Tables 2 and 3). Nevertheless, the overall number of mutations in non-tail-encoding genes detected in phages isolated on *waaG* compared to those isolated on WT *E. coli* was only 1.75-fold elevated (8 versus 14 clonal mutations) (Supplementary Tables 2 and 3). This elevation can be explained by the lower effective burst size of phages grown on the *waaG* host compared to phages grown on the WT strain, which thus undergo more generations to form a visible plaque. These results indicate that a general mutagenesis mechanism in the *E. coli waaG* host could not account for the observed EOP, which exceeded the expected EOP by several orders of magnitude.

Due to the aforementioned separation between the growth limitation and DNA replication, the apparent SIM can only occur either from a pre-existing mutation or from cryptic growth followed by selection. To rule out the former possibility, we used high-throughput DNA sequencing of the tail fibre gene (gene 17) of DNA isolated from emerging plaques. This type of sequencing allows the fraction of mutated genomes in a population to be examined. This technology thus enables the determination of whether an observed mutation pre-existed or was generated during plaque expansion. Each observed plaque originates from a single ancestor and so a mutation observed in all of the progeny can be considered a pre-existing mutation. In contrast, a mutation found in only a fraction of the progeny must have formed after plaque expansion, which in the current case occurred after exposure to the growth limitation (Fig. 1b). High-throughput DNA sequencing was carried out on polymerase chain reaction (PCR)-amplified DNA isolated from 30 different plaques grown on the restrictive host *E. coli waaG*, in two independent experiments. As Supplementary Table 1 shows, nucleotides G36241 and A36242 were found to encode a key residue whose substitution was the most frequent in allowing better recognition of the altered *E. coli waaG* host (see the adsorption efficiency of a mutated phage in these residues, $T7^{D540G}$, in Supplementary Fig. 1). We therefore analysed 10,000 to 200,000 genomic reads of these nucleotides. We detected a significant percentage of mutated genomes in these specific nucleotides in 15 out of 30 of the analysed plaques. These mutations were found in 1–96% of genomes, in most instances in less than 50%, but never in 100% of the genomes obtained from a single plaque (Table 1). Analysis of these nucleotides from 30 control plaques grown on WT *E. coli* showed that all of these genomes lack the mutations, as expected. These results indicate unequivocally that the emerging phages do not encode pre-existing mutations in these nucleotides before exposure to growth limitation. Therefore, these apparently stress-induced mutations arise during cryptic growth and are then selected by the growth limitation.

We have shown that pre-existing mutations that enable the phage particle to recognize the altered receptors are not the mechanism that allows phage growth. We therefore hypothesized that the non-mutated phage particle enables cryptic growth by allowing injection of DNA at low frequency, despite incompatibility with the host receptor. To show that this is indeed the case, we used a transduction system for T7 phage in which plasmids are packaged into the phage particle and transduce permissive hosts via adsorption and injection of the plasmid DNA (ref. 21). Transduction was monitored by counting colony-forming units (c.f.u.) that acquire an antibiotic resistance gene encoded by the transduced plasmid. We used these transducing particles to compare the transduction efficiency into restrictive (*waaG*) and permissive (WT) hosts. If the phage particle is able to inject DNA into incompatible hosts, regardless of phage propagation, then there should be a minimal difference in DNA delivery into the restrictive *waaG*

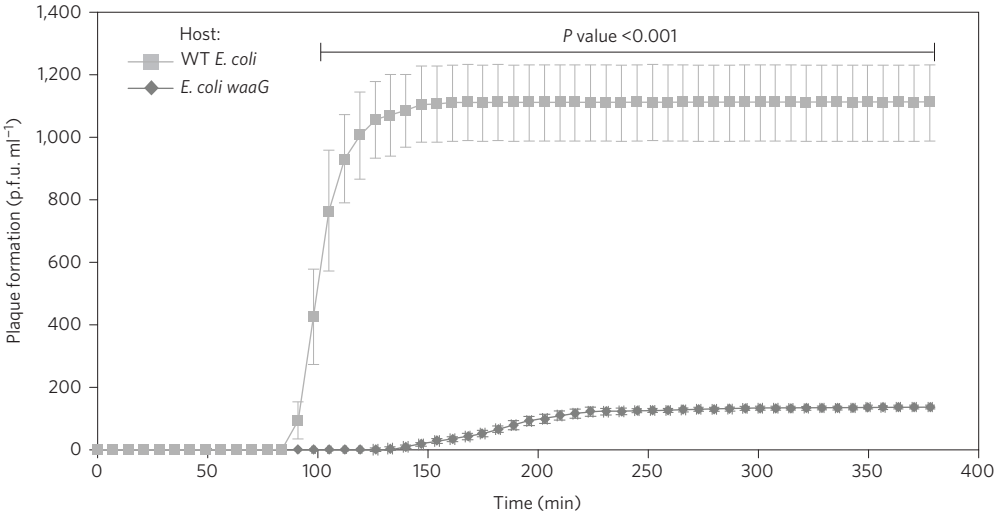

Figure 2 | Plaque formation of WT T7 phage on *E. coli* lacking *waaG* or on an isogenic *E. coli*. Plaque number was determined every 7 min. Points represent average number of plaque forming units (p.f.u.) ml^{-1} ± standard deviation of four independent experiments.

Table 1 | Percentage of mutant genomes at the indicated positions isolated from plaques of WT T7 bacteriophage grown on the *waaG E. coli* host.

Isolate number	Mutants at indicated position (%)	
	G36241	A36242
1	0	12
2	0	17
3	0	0
4	0	0
5	0	0
6	0	0
7	0	2
8	0	0
9	0	0
10	54	0
11	0	0
12	0	0
13	0	0
14	0	0
15	0	0
16	0	0
17	2	0
18	2	11
19	1	14
20	15	0
21	0	35
22	4	0
23	1	5
24	0	4
25	96	0
26	0	0
27	0	25
28	36	0
29	0	0
30	0	0

hosts between selectable DNA markers and phage DNA (Fig. 1c). We therefore expected it to inject the plasmid DNA into the restrictive hosts at a transduction efficiency comparable to the EOP of phage DNA injection on the same restrictive hosts. This efficiency should be similar provided that DNA elements such as the host promoters, known to facilitate injection[22,23], are absent from the infecting phage DNA. Indeed, a comparison of the efficiencies of a phage deleted for its host promoters with the efficiency of transduction of the encapsidated plasmid demonstrated similarity (P value = 0.07). The plating efficiency of phage D352-836 (deleted for ϕOL, A1, A2 and A3 promoters) on *waaG* hosts was 0.011 ± 0.006 times that of the WT *E. coli* host. The transducing particles showed a similar transducing efficiency on *waaG* hosts of 0.023 ± 0.006 times that of the WT *E. coli* host. This indicated that the receptor-binding protein is able to recognize the receptor of the restrictive host with decreased efficiency, highlighting the mechanism that allows cryptic growth until beneficial random mutagenesis occurs.

We present a biological system that demonstrates apparent adaptive mutagenesis by showing that mutations accumulate at frequencies that are significantly higher than expected following a specific growth limitation. We show that these mutations were not pre-existing, but rather emerged during growth limitation. The mechanism underlying this phenomenon is low-frequency DNA injection that enables cryptic growth until a selectable mutation is generated.

This model system is thus significantly different from bacterial systems monitoring mutations under sublethal conditions, because in those systems it is impossible to separate selection, DNA metabolism and possible mutagenesis. This example does not necessarily reflect the situation in more complex systems, but it does provide a unique example of a growth limitation condition, separated from the mutagenesis, under which mutations are selected for but not induced.

One may argue that the separation between growth limitation and mutagenesis is not complete, as the inefficient entry of the phage DNA could cause DNA damage or unusual replication due to physical blockage/shear forces/slow DNA entry into the cell favouring some degradation. Nevertheless, such scenarios would be accompanied by mutations in non-relevant genes due to general increased mutagenesis, which was ruled out by complete high-throughput DNA sequencing.

The receptor incompatibility of the restrictive host results in infection incompetency of most of the phages due to adsorption deficiency (Supplementary Fig. 1). Phage DNA injection into the restrictive host is successfully accomplished by a small portion of the phages. Once inside the host, the growth rate of these phages should be normal, as DNA replication does not depend on receptor incompatibility[16] (Supplementary Fig. 2). However, adsorption of the progeny remains inefficient, resulting in slow plaque formation (Fig. 2). Cryptic growth persists until a compensatory mutation(s) in the genes encoding the tail and tail-fibre proteins that overcomes the adsorption barrier is randomly generated. The mutated phages then

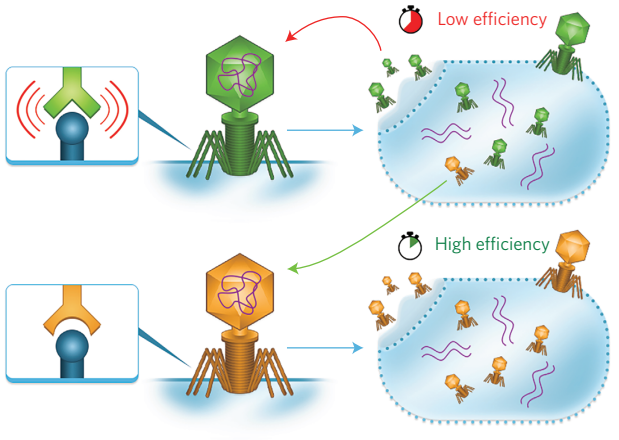

Figure 3 | Model depicting cryptic growth of the phage and selection. T7 phage infects a host with an incompatible receptor at low efficiency (growth limitation condition). Following DNA penetration, DNA replication and phage packaging proceed at normal efficiency with normal burst size. However, further infections by the progeny are inefficient due to receptor incompatibility. Random mutants that encode a compatible receptor-recognizing ligand eventually evolve and infect with high efficiency.

outgrow the parental phages because their adsorption is more efficient (Supplementary Fig. 1) and therefore the number of effective progeny is significantly higher (Fig. 3).

Our observations show a mechanism that allows the phages to co-evolve with resistant hosts having a mutated receptor and thus to broaden their host range. We show that phages can infect a host with an altered receptor, presumably to explore new hosts or altered resistant hosts. If successful, more progeny are produced, which increases the phage's chances of eventually acquiring a mutation that overcomes the restriction barrier. This study thus highlights an elegant solution for outsmarting resistant host mutants, in which no increased mutagenesis is required in the genome and thus there is no fitness cost or deleterious mutations associated with the selectable adaptive mutation, beyond those seen during normal growth. In the example presented herein, the fitness cost is merely a statistical cost for a small portion of individual phages, which might fortuitously infect non-supporting hosts, and is not a genetic alteration carried on permanently to the next generations. Most importantly, if no restrictive hosts are present in the environment, then no futile infection occurs.

This strategy demonstrates a novel way by which phages extend their host range via 'accidental' recognition of 'wrong' bacterial receptors. This allows the single phage to overcome growth limitations without mutations. If the growth limitation persists, then at the population level, the fittest phage acquiring beneficial mutation is naturally selected. These series of events only appear as SIM, but are actually driven by natural selection.

Methods

Reagents, strains and plasmids. LB medium (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl, wt/vol) was obtained from Acumedia, agar was from Difco and antibiotics were from Sigma-Aldrich. PCR amplification was carried out using Taq 2X LAMDA Master Mix cat# D123P (Lamda BIOTECH). Oligonucleotides were ordered from Hy Laboratories. The bacterial strains used were *E. coli* BW25113Δ*waaG* and BW25113Δ*ydhQ*. The phages used were WT T7 phage with a single variation in gene 12 from the sequenced T7 phage (T26921C, encoding Gp12-S694P) and phage D352-836 deleted for ϕ*OL*, A1, A2 and A3 promoters[24].

Kinetic monitoring of efficiency of plating assay. Overnight cultures of the indicated *E. coli* strains (0.2 ml; ~3×10^8 c.f.u.) were mixed with T7 phage (50 µl; ~50–1,000 plaque forming units (p.f.u.)) in 3 ml LB supplemented with 0.7% (wt/vol) agar at 42 °C. Suspensions containing the infected cultures were then overlaid on LB–agar plates. Plates were incubated on top of a digital scanner at 37 °C and automatically scanned every 7 min. Plaques were counted from the scanned plates at the indicated time points. Overnight cultures were used for convenience after determining that the EOP is similar on stationary and logarithmic growth-phase cultures.

Efficiency of adsorption assay. Overnight cultures of *E. coli waaG* or an isogenic control strain were diluted 10 fold in LB. These cultures (0.5 ml each; ~7.5×10^7 c.f.u.) were infected with ~3.75×10^6 p.f.u. of the indicated phages to yield a multiplicity of infection of ~1:20. The infected cultures were incubated 7 min at 37 °C and then placed on ice. One sample taken from the culture was left untreated to determine the total phage count. Another sample was treated with chloroform to determine the number of non-infecting phage particles. Serial dilutions of each culture were spotted (15 µl) on LB–agar plates containing wt-*E. coli*. The plates were incubated at 37 °C and plaques were counted 16 h after infection. Efficiency of adsorption was calculated by subtracting the number of plaques counted on the chloroform-treated samples from the total plaque number and dividing the difference by the total plaque number.

Efficiency of centre of infection (ECOI) assay. Overnight cultures of the indicated *E. coli* strain (0.5 ml each; ~7.5×10^8 c.f.u.) were infected with ~3.75×10^6 p.f.u. of the indicated phages to yield a multiplicity of infection of ~1:200. Infected bacteria were then centrifuged for 1 min at 13,000*g* at 4 °C. The pellet was suspended in ice-cold LB supplemented with 1 M NaCl, transferred to a fresh tube, and centrifuged again to wash residual non-infecting phages. This washing step was repeated twice more and the pellet was then suspended in 0.5 ml ice-cold LB. Serial dilutions of each culture (15 µl) were spotted on LB–agar plates containing WT *E. coli* or *E. coli* lacking *waaG*. The plates were incubated at 37 °C, and plaques were counted 16 h after infection. The ECOI was determined by dividing the number of plaques obtained on each bacterial lawn by the number of plaques obtained on the WT *E. coli* bacterial lawn.

Efficiency of transduction assay. Overnight cultures of the indicated *E. coli* strains were diluted 1:100 and grown in LB at 37 °C to an optical density at 600 nm of 0.5–0.8. Cultured bacterial suspensions (100 µl) were mixed with 50 µl T7 phage lysate containing 50–1,000 transducing particles in 3 ml of 42 °C LB supplemented with 0.7% agar. After incubation for 5 min at room temperature, suspensions were spread on LB–agar plates supplemented with 100 µg ml^{-1} ampicillin and incubated at 37 °C. Colonies were counted 16 h post-infection.

Plaque purification for DNA sequencing. Plaques emerging on the indicated hosts were picked and purified by two sequential platings on the same host strain. PCR using primers 162F: 5′ -AAGAAACTCGATACACTATC-3′ and IY176R: 5′-GTCGAGCACTAACATCTGCT-3′ was carried out as follows: 5 min at 95 °C, 30 cycles of 20 s at 95 °C, 20 s at 56 °C and 100 s at 72 °C, followed by 200 s at 72 °C. Products were treated with Exo-Sap and then sequenced using IY176R: 5′-GTCGAGCACTAACATCTGCT-3′.

High-throughput sequencing. To generate a large number of phage genomes for high-throughput sequencing, plaques grown on BW25113Δ*waaG* or BW25113Δ*ydhQ* were picked into 0.5 ml LB. Two drops of chloroform were then mixed thoroughly into the solution. The chloroform was sedimented by centrifugation at 13,000*g* for 2 min. A 1 µl aliquot of the solution was used as the template in a PCR using primers 5′-NNNNXXXTCCGCTTCCGCAATATCTGG-3′ and 5′-NNNNXXXCTCCACCATGATTGCATTAG-3′ (XXX represents the three-base barcode specific to each plaque). These PCR products were further manipulated using the Illumina kit (catalogue no.15025064, barcode 3) according to the manufacturer's instructions. Sequencing was carried out using Illumina's HiSeq 2500 in a rapid-mode single-read run.

To determine the occurrence of mutations in the entire genome, we isolated ten WT T7 plaques grown on BW25113Δ*ydhQ* and ten mutant plaques grown on BW25113Δ*waaG*. Each plaque was streaked three times on LB–agar plates overlaid with soft agar containing 0.2 ml of the respective host bacteria. Equal phage numbers from ten different WT T7 or mutant plaques were each pooled, and genomic DNA of the phages was extracted from these cultures. Sequencing was carried out using Illumina's HiSeq 2500 in a rapid-mode single-read run.

Bioinformatics analysis of mutation rate. Sequencing reads were divided into the different barcodes and primers (Fwd/Rev) based on their first 8 bp. Analysis was performed separately for each barcode and primer. Low-quality and irrelevant reads were first discarded, and we only used reads that fulfilled the following criteria: (1) the read contained exactly 150 bp without Ns, (2) the mean Phred quality score was ≥30, and (3) the read had up to 20 mismatches against the amplicon template. For each position, the mutation ratio was calculated by counting the number of reads with a mutation in the given position divided by the total number of high-quality reads. We noted positions that consistently demonstrated relatively high mutation rates across independent samples (barcode and reads).

To analyse mutation frequency in the mixtures of ten isolated plaques grown on BW25113Δ*ydhQ* or BW25113Δ*waaG*, we first filtered reads based on quality. Only reads with quality score higher than 20 (probability of sequencing error <0.01) were

used in mutation frequency analysis. Reads were mapped to the T7 genome (NC_001604) using BWA aligner[25]. The resulting sequence alignment/map output was analysed using a Perl script based on calculation of the number of matches and mismatches to each genomic position.

Statistics. Unpaired *t*-test was used to determine *P* values.

Accession codes. Sequencing data have been deposited in NCBI Bioproject database under accession no. PRJNA314727.

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Author contributions

I.Y., R.E. and U.Q. conceived and designed the experiments. I.Y., R.E. and G.A. performed the experiments. I.Y., R.E., A.L., R.S., A.M. and U.Q. analysed the data. U.Q. wrote the manuscript with input from all authors.

Additional information

Supplementary information is available online. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to U.Q.

Competing interests

The authors declare no competing financial interests.