Fitness in bacteriophages

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Summary

Bacteriophages are viruses that infect bacteria. Since their first description in 1915 a myriad of studies investigating numerous topics of molecular biology were carried out using bacteriophages. In addition to groundbreaking discoveries in the fields of genetics and virology, bacteriophages were also used to investigate the role of mutations and their influence on fitness and evolutionary progress. The purpose of this review is to give an overview of the insights gained about fitness studies in bacteriophages, particularly focusing on fitness studies using the plaque assay technique and mathematical modelling on the propagation velocity of said plaques, as well as direct comparisons of fitness by competition assays of different phages. Furthermore, discrete episodes of the phage life cycle shall be analysed in the context of fitness states.

Contents

Introduction

1. Phages

Bacteriophages were first described by Twort in 1915[1, 2] and by d'Herelle in 1917[2, 3] as an infective agent capable of lysing bacteria. Ever since then, a vast amount of research has been conducted, regarding numerous topics concerning bacteriophages, e.g. genome, structure of the virus particle, infectious cycle (lytic and lysogenic), possible therapeutic applications, evolution of phages et cetera. Bacteriophages are viruses that infect bacteria. They are divided into 13 families and an unassigned genus[4] (appendix I). The structure of different bacteriophages can vary extensively[4]. However, there are characteristic features that most phages display (Fig. 1)[66]. The head or capsid is composed of proteins and contains the genetic material, which can be either DNA (single-stranded (ss) or double-stranded (ds)) or RNA (ss or ds), although most phages contain dsDNA. More than 95 % of phages also possess a tail structure[4], which serves to inject the genetic material into the host cell. The tail can be further attached to tail fibers and an end plate, which enables the binding to the bacterium[5].

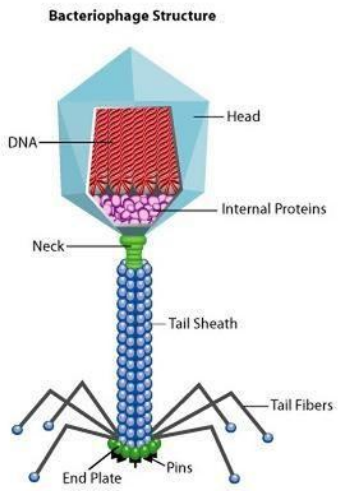

Figure 1. Schematic illustration of a phage, taken from[66]. The general features of a bacteriophage are shown: head containing genetic material (e.g. DNA), tail structure allowing injection of phage DNA into the host cell and end plate and tail fibres involved in binding process of the phage to the bacterium[5,66].

The lytic life cycle of phages (the lysogenic life cycle shall be disregarded for this review) involves three important stages: adsorption of the virus particle to the host cell, maturation of new virus particles in the host and lysis of the host in order to release the newly produced progeny (Fig. 2)[67]. For an infection to occur, the phage has to absorb to the surface of a susceptible bacterium, via receptors on the host cell[6-8]. Adsorption of the virus particle to the host cell is followed by the injection of its genetic material into the bacterium and using the host's synthesis machinery and resources to generate new virus particles. Assembly of new phages takes place after a certain period of time, referred to as the eclipse period[8]. Before lysis of the bacterium can occur, lysis proteins, which are encoded by the phage, need to be expressed[9-11]. The length of the intracellular phase of the phage lytic life cycle is also referred to as latent period. The number of particles released through lysis of the bacterium is referred to as burst size or phage fecundity[2]. Although it was shown that the burst size is not influenced by the size of the bacterium[12] there are several factors that determine the burst size of an infectious cycle, as shall be discussed later in this review.

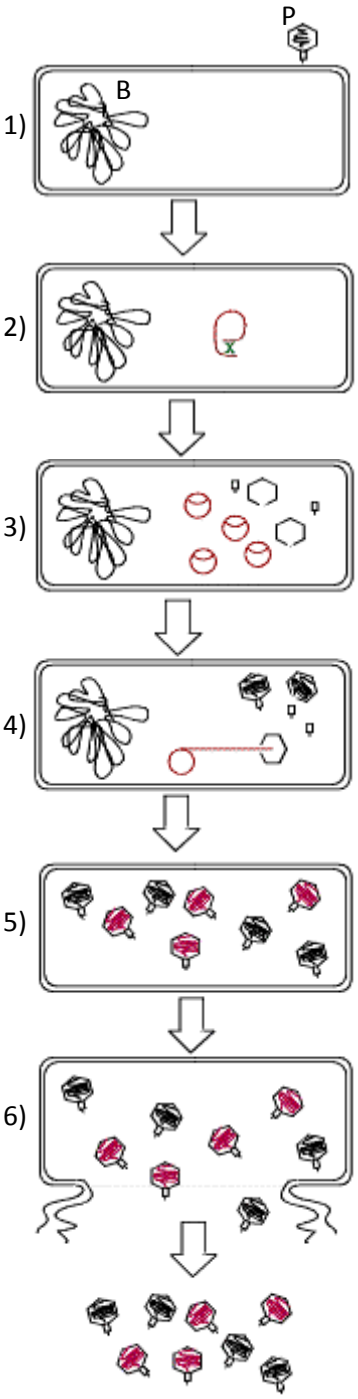

Figure 2. Schematic representation of bacteriophage lytic life cycle, taken from[67]. Phage (P) absorbs to the surface of a susceptible bacterium (B) (1). Genetic material of phage is injected into the bacterium (2). Phage uses synthesis machinery and resources of host cell to produce viral proteins (3). Replication of viral genome and assembly of new virus particles (4). Accumulation of virus particles and expression of viral lysis proteins (5). Lysis of the host cell and release of phage progeny (6).

In conclusion, the central life-history traits of the lytic life cycle of bacteriophages are adsorption, latency and lysis (including burst size). As this life cycle is comparatively simple, bacteriophages are often used for theoretical studies, including optimality models, evolvability and trade-off relationships among life-history traits[8].

2. Fitness and evolution

In order to gain a better understanding of the concepts used to investigate fitness differences in phages, it is useful to consider general approaches and theories regarding fitness and evolution. Fitness can be defined as the 'overall replicative and survival capacity of an organism in a particular environment'[13]. Changes of this fitness status are a consequence of mutations in the genome, which occur spontaneously as the driving force for evolution[14]. However, mutations (variations of the genotype) do not necessarily lead to differences in the phenotype of the organism, which is illustrated by the occurrence of neutral mutations. It is a highly complex problem to estimate the effect of a certain mutation on the fitness of an individual or a population, as numerous parameters have to be taken into consideration. It becomes even more complex when including the evolutionary role of the mutation, as short-term fitness advantages (adaptation to temporary fluctuations of the environment) are not necessarily a positive progression in the long-term. The evolutionary impact of a mutation is characterised by the genomic mutation rate (*U*) and the distribution of the fitness effects of the mutation (*f(s)*), which alternate not only among species and environments but also within species[14]. This introduces another variable, the environment. Differences in natural environments are usually qualified in terms of varyingly stressful conditions, e.g. imposing metabolic constraints on the organism[14]. Notably, displaying fitness gains under stressful conditions could simultaneously result in fitness losses under less extreme conditions[15]. Furthermore, it was observed that the extent of variation of genomic mutation rates of microorganisms possessing DNA genomes is not nearly as vast as expected considering the genome size[16]. It was found that even in extremely stressful environments the genomic mutation rates are fairly constant, which is remarkable given that mutations are partially caused by DNA damages, which are elevated under these conditions[16].

Overall, it is evident that fitness variations and their impact on evolution are a very complex subject matter. In an effort to visualize fitness Wright introduced the notion of fitness landscapes in 1932, a multidimensional landscape mapping the genotype against its respective fitness[17, 18]. Classically, an optimal fitness solution is represented as a global maximum, which can be surrounded by suboptimal fitness solutions (local maxima) (Fig. 3(a))[18]. Focusing on a distinct genotype and introducing a mutational operator results in a cluster of possible offspring genotypes, which can display higher, lower or similar fitness than the parental genotype. The concept of fitness landscapes also illustrates the effects of neutral mutations of evolving populations, moving in one dimension of the landscape (genotype) but staying at a constant height (fitness) (Fig. 3 (b and c)). This can be described as random drifting of the population, which ultimately might enable a population to escape from a local fitness optimum (Fig. 3 (b and c))[18]. Another focus in this context is the concept of quasispecies, which was introduced in 1978 by Eigen and Schuster[19, 20]. It also regards the drifting character of neutral mutations and their impact on selective evolution. The basis of this concept is the stochastic theory by Kimura[20, 21], which hypothesizes that mutations are statistically rare events compared to the large size of the genome and a limited population number[20]. However, this does not hold true for viral genomes, as individual mutants can occur reproducibly, which is particularly true for neutral mutations[20]. This does not lead to a population of a single wild-type but a 'distribution of mutants that belongs to the maximum eigenvalue of the system', which is referred to as a quasispecies[20].

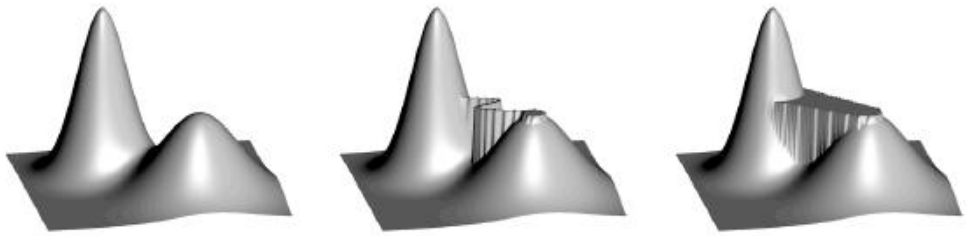

Figure 3. Exemplary multi-dimensional fitness landscape displaying a global and a local optimal fitness solution (a), taken from[18]. Examples of drifting populations caused by neutral mutations, which allow populations evolving within the local peak to cross to the global peak, in a limited manner (b) or more permissively (c)[18].

A last aspect to introduce regarding fitness is the concept of evolvability, or the capacity of individual (or population) to evolve by generating fit variants[18]. Evolvability describes the potential for fitness, i.e. equally fit individuals can have different evolvabilities[18].

Fitness in bacteriophages

3. Plaque assay

    a. Method

One of the standard techniques to measure fitness in bacteriophages is the nutrient agar layer method, which was first described by Gratia to enumerate phage particles[2, 22]. A thin layer of soft agar, containing host bacteria and bacteriophages, is poured on a thick layer of higher concentrated agar, which serves as nutrient medium for the bacteria[2]. After adhesion of the phages and infection of the bacteria, new phage particles are produced within the bacteria and released upon phage-induced bacterial lysis, starting a new infectious cycle. The area, in which the bacteria are infected and finally lysed, has a clear circular shape (plaque) and is distinct from the turbid bacterial lawn. Plaque shapes and sizes might differ depending on the agar used for the nutrient layer and on the media used for the overlayer[23, 24]. The concentration of the agar can also have an effect on number and size of the plaques[24-26]. The age of the host bacteria was not found to influence the number and/or size of the plaques, but the concentration of available host cells is of relevance[24]. In newer studies, it was suggested that the traditional nutrient agar base can be omitted when adding the nutrients directly into the plating agar[27]. This way an additional parameter for mathematical modelling could be excluded[27].
Very early on it was shown by Ellis and Delbrück[28] that the plaque count is proportional to the relative concentration of phages used for infection, indicating that a single particle is responsible for the formation of a plaque, assuming that virus particles are spread out among host cells according to a Poisson distribution[29]. This hypothesis is further sustained when considering that the observed ratio of infected : total particles for bacteriophages is in line with estimates for the single-particle

concept, while it differs from a concept where two particles would be needed to cause an infection[29].

b. Plaque types

Plaques produced by phages can vary in size and shape (Fig. 4). Plaques can be circular, irregular, fuzzy, sharply defined, clear, surrounded by halos, etc[23, 25, 30] (Fig. 4). It was shown that some phages, e.g. MT_2, which grows on various mycobacteria, are able to form different types of plaques: large plaques that are surrounded by a halo or small plaques that have no halo[25]. It was excluded that these plaques were produced by two different phages which both would have been able to infect the same mycobacteria species and it was speculated that a spontaneous mutations could be the cause for the occurrence of the small plaques[25]

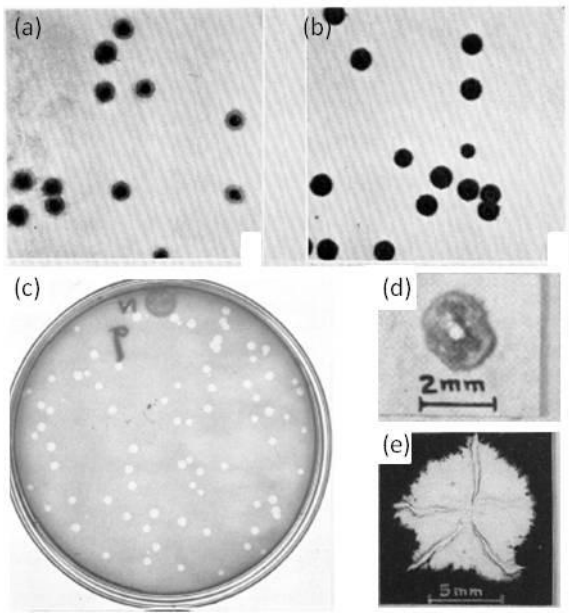

Figure 4. Representative images of plaque types and sizes, taken from[31,23,30]. Plaques of wild-type T2 phage, which are surrounded by pale halos[31] (a). Plaques of *r* mutant T2 phage, displaying clearer halos than the wild-type[31] (b). Halo-less circular plaques of T3 phage at 4 hours of incubation[23] (c). Different streptomycete phages at 48 hours of incubation[30]. Plaque surrounded by halo with irregular margin[30] (d). Large turbid plaque of irregular shape[30] (e).

c. Advantages and disadvantages

There are several advantages of the plaque assay system compared with alternative methods to measure fitness of bacteriophages. Plaque assays are relatively inexpensive, easily implemented and results are quickly obtained. One of the main assets of this system is that a single virus particle infecting a single host cell is giving rise to the plaque, allowing to trace events in a time-dependent manner[27]. This also allows using plaques in order to purify fairly homogeneous samples of particular phage strains[32]. Furthermore it is noteworthy, that the conditions during a plaque assay are a better representation of natural circumstances, under which bacteria get infected with bacteriophages, rather than replication assays in liquid culture or serial transfer experiments[33]. This is based on the fact that bacteria in their natural environment often occur in biofilms, which is mimicked by the bacterial lawn.

On the other hand it is difficult to supply fresh host cells for the replicating phage while using the plaque system. Moreover, there have been reports of difficulties in replicating results[34]. However, it seems that with increasing accuracy of the techniques this issue is solved.

4. Genetic factors

    a. Plaques and differences in fitness

Differences in fitness of bacteriophages, as of any other organism, are caused by mutations of the genome, which can be beneficial or detrimental. These mutations can have a variety of effects, e.g. changing the host range of the bacteriophages or causing differences in their growth rates[35]. Mutants that differ in their fitness from the wild type can be tracked on plaque assays. An example for this is the 'star' mutant of phage T2 which was discovered by Hershey and Visconti in 1953[31, 36]. 'Star' mutants or S mutants are phages whose plaques have a distinct morphology. The centre of these plaques is surrounded by a small halo which is interrupted by spots of rapid lysis resulting in a segmented appearance[31] (see Fig. 5). It was shown that the 'star' mutant of phage T2 has a single mutation at locus s_1 which is in proximity to locus r_1. This mutation is not disadvantageous for the phage when infecting young bacteria. However, when grown on old bacteria, the s mutant was found to have an extended latent period and its progeny re-absorbed considerably slower to new host bacteria. The observation that the age of the bacteria is of relevance for the development of the plaque is a distinct characteristic of the star mutant. It was proposed that these findings could explain the distinct morphology of the 'star' phages. In the beginning of the culture, when the bacteria are young, the multiplication rate of the s mutant won't be inhibited and several spontaneous mutations occur. These mutations can be neutral at that moment. However, with increasing age of the bacteria, which results in decreasing fitness of the star mutant, the new mutants can have a selective advantage, even if they are only able to multiply at 'normal' rate. As the mutants outgrow the star phage at the border of the plaques they form their distinct morphology[31].

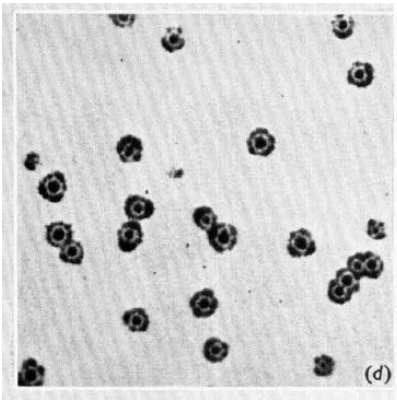

Figure 5. Photograph of coliphage T2 star mutant (s_1r^+) plaques, taken from[31]. These display an irregular halo, which is characteristic for the star mutant[31].

Another example to illustrate the effect of mutations in the plaque assay system was given by Yin[37]. A growing plaque of a T7 bacteriophage was stab sampled along different radii as shown in Fig. 6[37]. Therefore the plaque was stabbed with a sterile pipette tip at a determined position, which was subsequently washed with phage buffer. This process was repeated for several positions as shown in Fig. 6 and the obtained phage samples were used for genotyping. The original phage had been grown

on BL21 *E. coli* bacteria for 50 h, which express the T7 RNA polymerase. With increasing distance to the centre of the plaque, mutants of the wild type phage were detected that had deleted part of their genomes, which included the RNA polymerase gene. When testing the mutants in one-step growth cultures as well as in competitive plaque assays against the wild type phages, it was found that the mutants grew faster and had higher propagation rates on BL21 *E. coli*, indicating a significant increase in fitness. It is noteworthy, that the mutants could only be detected with a certain distance from the centre of the plaque, demonstrating that adaptation to the novel environmental conditions calls for a certain amount of time. Yin further suggested that the emergence of mutations increases with higher host concentrations. However, it is not clear whether this implies that these conditions are a favourable mutagenic environment or if the increase in host cells simply increases the number on phage progeny and therefore the likelihood of mutations.

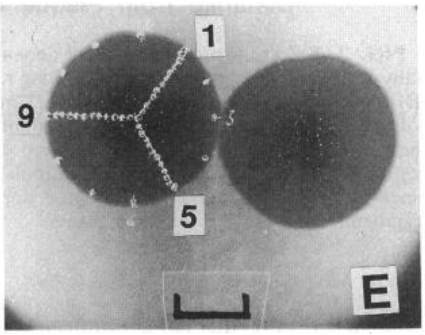

Figure 6. Exemplary image of sampling a bacteriophage T7 plaque along three radii (1, 5, 9), taken from[37]. Phages were incubated for 50 hours on BL21(DE3) bacteria, which express T7 RNA polymerase. Phage samples that were more distant from the plaque centre displayed higher propagation rates[37].

Finally, it was even observed that a mutant, resulting in a faster growth rate of the phage, is capable of changing the morphology of the plaque, introducing asymmetries (Fig. 7(a))[38]. It was further shown that the mutant when isolated from the original plaque grew 60 % faster (Fig. 7(b))[38].

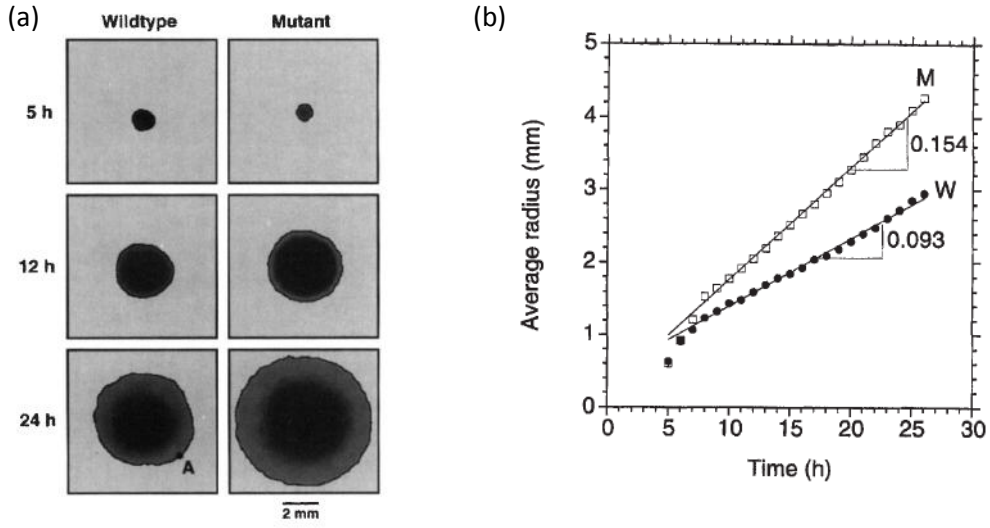

Figure 7. Images of bacteriophage T7 plaques after 5 h, 12 h and 24 h of infection, taken from[38]. Left panels show plaques initiated by wild-type phage. Right panels show plaques initiated by mutant phage A, which was isolated from wild-type plaque[38] (a). Plaque growth rates of T7 wild-type phage (W) and T7 mutant phage (M)[38] (b).

b. Limits of adaptation

Adaptation and an increase in fitness via mutations of the genome are not limitless processes. To determine whether certain features of the genome set limits to adaptation in bacteriophages Bull *et al.* compared eight different bacteriophage strains that were grown under the same rapid growth conditions[39]. Fitness was measured as the number of doublings of the phage concentrations per hour in a liquid culture, and therefore taking into account that generation times between the phages varied. Of the eight bacteriophages which were adapted to the same environment, two were always closely related. Two phages with small RNA genomes, two phages with small single-stranded (ss) DNA genomes, two phages with moderate-sized double-stranded (ds) DNA genomes and two phages with large dsDNA genomes were analysed. This allowed to examine whether possible differences in fitness limits were due to global constraints (rate-limiting host processes) or local constraints. When comparing starting fitness with the final fitness of each phage, it was found that the moderate-sized dsDNA phages were able to increase their fitness to a significantly higher extend than the three remaining phage types (Fig. 8)[39]. The limitations in gaining fitness varied seven orders of magnitude. This indicates that fitness limits are not set by a high mutation rate or a small genome size given that the phages with an RNA genome were not able to increase their fitness significantly. Bull *et al.* argue that fitness limits are rather set by the ability of the phage (or its genome composition) to overcome rate-limiting host processes, e.g. transcription and translation, suggesting that fitness limits are set by global constraints. They further note that the measured fitness gains are potentially irrelevant in a natural environment, given that the phages used for this study were cultivated in the laboratory for several decades[39].

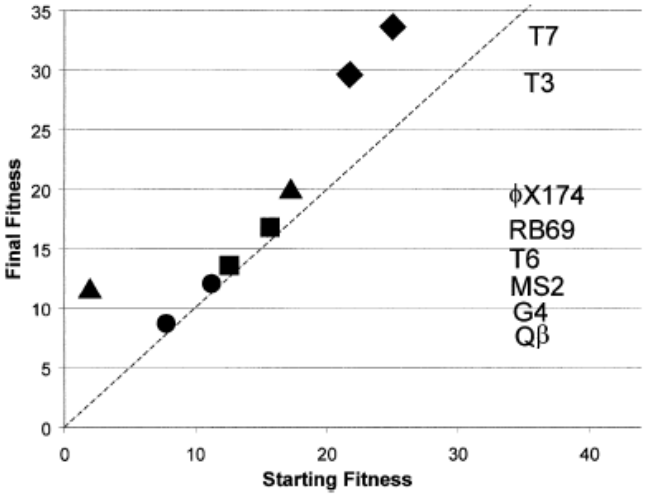

Figure 8. Fitness adaptation of different bactiophages, taken from[39].Starting fitness was plotted against final fitness, dashed line illustrates no fitness variance at all. 4 phage groups were analysed: RNA phages MS2 and Qβ (circles), ssDNA phages G4 and ϕX174 (triangles), dsDNA phages RB69, T6 (squares, large genome), T3 and T7 (rhomboids, moderate-sized genome). Phage names are listed in order of final fitness[39].

c. Mutation rate; Muller's ratchet

Fitness of phages is also affected by the mutation rate of the virus. Muller hypothesized in 1964 that in an asexual population with a high mutation rate the number of mutation-free individuals would be bound to become increasingly smaller, with no prospect of restoring mutation-free individuals as recombination of genes could not occur and back-mutations are rare events[40, 41]. This irreversible process is referred to as Muller's ratchet. The non-segmented RNA bacteriophage ϕ 6 is effectively

asexual and was used to determine whether detrimental mutations would accumulate and fitness of the bacteriophage therefore decrease[41, 42]. 40 cycles of plaque-to-plaque transfers were carried out and clones of each cycle were preserved. Using a genetically marked wild type clone, paired-growth experiments with the wild type and the transferred clones were carried out and mean fitness was calculated. Fitness differences were determined as the relative variation of the transferred : parent clone ratio. It was found that transferred clones in some cases were able to increase their fitness by 6%, while other clones suffered a fitness loss of up to 71%. The mean fitness of the transferred clones compared to the parent clone was calculated to be 0.78, indicating a considerable drop in fitness. It was further noted that although back mutations can occur, the incidence rate is not high enough to compensate for the high mutation rate and therefore cannot stop Muller's ratchet[41].
A different study with a similar intent was carried out by Springman *et al.* in 2009[43]. In order to investigate the load of a high mutation rate on the fitness of a population, bacteriophage T7 was cultured in an artificial mutagenic environment and its fitness variance determined. Bacterial broth cultures, supplemented with a mutagen, were infected with phages and passaged for 200 generations. Fitness was determined by the number of doublings per hour of the phage population. Surprisingly, fitness was found to have increased from 18.3 doublings/hour of the first generation to 21.9 doublings/hour of the 200th generation. This is in sharp contrast to the theoretically predicted fitness loss. When investigating possible explanations for this discrepancy, it was found that the mutation rate did increase, but that missense mutation were far more likely to occur in nonessential genes than in vital ones. It is suggested that a possible explanation for these observations might be an induction of adaptive evolution as a result of the high mutation rate[43]. However, as possible calculation inaccuracies are also indicated, these insights should be cautiously evaluated.
These examples show that although mutations are the basis of adaptation and allow phages to increase their fitness in some cases, it has to be a balanced system, which can recover when necessary.

5. Life cycle

As discussed earlier, there are three main life-history traits of the phage life cycle: adsorption, latency and lysis. Each of these traits can be studied in relation to fitness advantages or disadvantages.

a. Adsorption

The time period of finding and attaching to the host cell (t_s) is defined by the density of the host cells and the rate of adsorption (r) of the phage[8]. This suggests an implicit equality of the two as a high host concentration can compensate for a low adsorption rate in the search of a host bacterium by the virus and vice versa[8].
Host recognition by the pathogen was found to obey the law of mass-action (describing solutions in a dynamic equilibrium) with respect to the concentration of host bacteria and the concentration of free phage particles, which was first described by Krueger in 1931[44]. For host recognition by the pathogen the equilibrium is shifted in favour of the adsorption[28]. This is still the case when the phage concentration is higher than the concentration of host cells, suggesting that several phages can attach to a single bacterium[28].

There are several studies demonstrating the influence of the adsorption rate on the fitness level of bacteriophages. In a study by Shao and Wang competition assays between genetically marked (l*acZα*$^{+}$ and *lacZα*$^{-}$, see also competition assays p. 20) isogenic phage λ-strains which differed in their adsorption rate were carried out[8]. Relative fitness of the competing phage strain was determined by counting the emerging clear and blue plaques separately after 4 hours of infection. It was found that the phage strain with the high adsorption rate had a shorter optimal lysis time (46.0 min) than the phage strain with the low adsorption rate (64.0 min) resulting in a higher relative fitness of the high-adsorption strain because of a faster growth rate due to the shorter lysis time. In continuation of this study similar experiments were carried out in an environment mimicking bacterial biofilm (agar-entrapped bacteria[45]) in order to investigate the impact of the adsorption rate under more natural conditions[46]. Three genetically isogenic λ phages with different adsorption rates were used to investigate settlement of the phages, as in the previous study, but in addition the size of the resulting plaques was also determined. It was found that, although it is advantageous under liquid culture conditions, a high adsorption rate might be disadvantageous for the propagation of plaques[39, 46, 47]. It was reasoned that a high-adsorption phage has a higher rate of settlement on the bacterial agar, but is not able to diffuse further because of this high affinity to the host cells[46]. Therefore, under a biofilm-like environment, a low adsorption rate seems to be advantageous, taking into account, that the concentration of available host cells can influence this correlation[47].
In the context of limited adaption of phages, that was mentioned earlier, adsorption can be regarded as an important global constraint as phage attachment depends on host-encoded receptors[39]. While investigating the relevance of genomic characteristics in limiting fitness gains, Bull *et al.* also measured the adsorption rates of all phages. It was found that, although adsorption by itself is not responsible for the determined fitness differences, a higher adsorption rate was linked to higher fitness gains when comparing related phages[39]. This effect is only valid in the context of fitness gains under liquid broth conditions.

b. Latent period, lysis and burst size

Although latent period and lysis (including the burst size) are different life-history traits of phages, they affect each other substantially and therefore shall be discussed together. The period of time the phage needs to complete the intracellular phase of the life cycle is called latent period, whose duration controls the eventual burst size as well as the generation time (and therefore to a certain extent the growth rate)[8, 48]. This subject has been investigated in numerous studies of bacteriophages[8, 48-52]. Interestingly, it was found early on that initiation of lysis is not provoked by a shortage of host cell resources[53, 54]. While the generation time will be shorter if the latent period is shorter, the burst size will be larger if the latent period is longer. The antagonism of these benefits results in an optimal latent period depending on the conditions present[11, 39, 50]. The latent period can be regulated by the phage by delaying host cell lysis, which is in general controlled with holin complexes (phage encoded proteins), but it also depends on the host physiology and its quality[48]. Furthermore, it was contemplated that an extension of latency could also be achieved by favouring lysogeny over the lysis[48, 55]. In this case the prolongation of the latent period is caused by an extension of the eclipse phase and not by extending the period of progeny maturation[48]. It was found that the occurrence of lysogeny was more probable when phage multiplicities exceed 1, i.e. under low host cell conditions[48, 55]. Several studies suggested[51, 56] that phages significantly adapt the

duration of their latent period to the quantity and quality of available host cells. When validating these findings, using a more elaborate modelling strategy than before, Abedon *et al.* proposed that the extent to which adaption occurs is smaller than previously assumed[48, 49]. This becomes particularly apparent under low host density conditions[48, 49]. It was suggested by Shao *et al.* that this is due to the fact that in the model the approximation of the average search time (before a phage encounters a new host cell) is no longer valid under low host densities, as deviations from the average have a larger impact[8]. Nonetheless, the general trend of selection for a longer latency period because of lower host cell densities was still confirmed by modelling results as well as experimental data[8, 48]. It is further relevant to see that adaption of the latent period, by delaying lysis in response to variances in host cell densities, does not inevitably lead to a higher growth rate of the phage. Other factors which have to be taken into account as well are the adsorption rate and possible variations of the eclipse period[48]. Indeed, a later study by Wang *et al.* predicted that duration of the optimal lysis time would be shorted under high-affinity adsorption conditions[50].

6. Growth rates (Plaque propagation)

In order to validate differences in fitness between bacteriophages, it is of interest to analyse plaque assays not only qualitatively but also quantitatively. Research concerning plaque growth rates of phages was already carried out in early studies using bacteriophages[24, 57]. In later studies mathematical models were developed and refined.

In an early paper by Luria[58] it was suggested that the plaque size is inversely correlated to the size of the phage. This correlation was soon dismissed by St. Clair and McCoy[30] reporting that in some cases the same phage is able to produce differently sized plaques depending on bacterial strain it has been grown on. Mayr-Harting made a number of substantial observations at the same time[24]. It was found that the diameter of plaques increased almost linearly in size for 15-20 hours after which it became slower until it eventually stopped (Fig. 9(a)). Furthermore, it was shown that plaques can fuse when in close contact and that if grown under same conditions, on the same host cells, plaques will reach the same size unless they fuse with another plaque. Notably, the number and the size of plaques were found to depend on the concentration of the host cells, even showing that a maximum number of plaques occur at a distinct host cell concentration. The first study focussing on the quantitative aspect of plaque development was carried out by Koch in 1963 using the T4 phage on *E. coli*[57]. The development of a plaque was divided into four phases: primary adsorption event, first rounds of multiplication, enlargement phase and the final phase, resulting in the slow halt of multiplication. Koch focused on the last two phases of this process, which mainly consist of infection, lysis, re-infection. However, the whole process is much more complicated, as the kinetics of diffusion, adsorption, viral growth and host cell growth have to be considered at the same time[57]. As Mayr-Harting observed before[24], Koch also found a linear increase of the plaque diameter in time, which ceases gradually over time[57]. Furthermore, he stated that the plaque size appears to be inversely related to the latent period of the phage[57]. The model of plaque growth rate suggested was mainly determined by two parameters, the diffusion rate and the latent period[57]. The assumptions were the following: plaque size is independent of the growth rate of the bacteria, phages spread to susceptible host cells by diffusion, i.e. plaque enlargement is caused by diffusion and the growth rate of the phage, the enlargement process occurs in waves. Focusing more detailed on the diffusion process he stated that given that the concentration of diffusing particles falls off quickly with distance from their

source, diffusion can be regarded to occur in one dimension from the region of high concentration to the region of low concentration. Diffusion was said to be rather slow, taking twice as long as the latent period and the covered distance would be small compared to the plaque size. The formula he developed[57] illustrated the dependence of the growth rate from the diffusion constant and the latent period,

$$C = a\,(D/L)^{1/2} \qquad \text{(equation 1, taken from[32])},$$

C being the radial propagation velocity of the plaque, D being the viral diffusivity, L being the latent period and a being a constant used to take reversible and irreversible viral binding into account. As mentioned before parameters as adsorption rate and burst size were neglected and the conclusion was drawn that the plaque enlargement rate was primarily dependent on the square root of the diffusion constant and inversely on the square root of the lag period.

(a) (b)

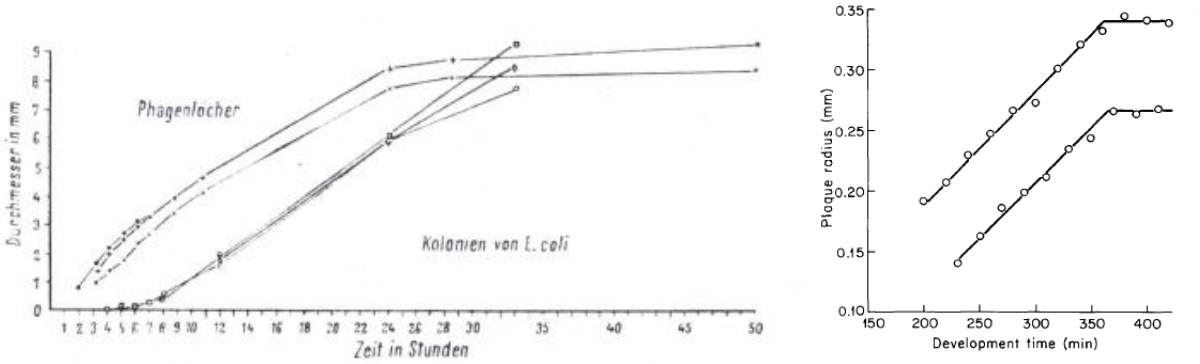

Figure 9. Bacteriophage plaque diameters plotted against time after infection, taken from[57,59]. Plots were obtained from different studies: Mayr-Harting[57] (a), Kaplan[59] (b). Plaques increase in diameter linearly before a decrease in the enlargement rate is observed.

A different approach to the subject was carried out by Kaplan *et al.* in 1981[59]. The average plaque size was determined as a function of the initial density of host cells, evoked by the observation that at high values of host cells plaques were not big enough to be accurately measured. It was assumed that the average surface area of a plaque can be determined by $3\pi r^2$ (describing a spherical shape that is truncated at the surface) and that the radius of a plaque increases linearly with time. This was tested using time-lapse photography of forming plaques (Fig. 9(b))[59] and in line with previous findings by Mayr-Harting and Koch, as described above[24, 57]. Furthermore, it was proposed that uninfected cells grow exponentially in soft agar, described by:

$$D = D_0 e^{\lambda t} \qquad \text{(equation 2)}^{59},$$

with D being the density of cells at time t, D_0 being the cell density at $t = 0$, and λ being the growth rate constant of cells under these conditions. Finally, it was assumed that plaques reach their final radius R at time t_R because the host cell population reaches a fixed density D_f, precluding further phage replication. Combining all assumptions, a function was proposed, in which the final radius R of a plaque decreases logarithmically with increasing D_0:

$$R = (k/\lambda)\ \ln\,(D_f/D_0) \qquad \text{(equation 3)}^{59},$$

with k being a rate constant for plaque growth.

In order to be able to calculate the total number of lysed cells in a plaque, the number of lysed cells at the surface area of the plaque was determined. To do so, the total time needed for the plaque to establish its final size had to be taken into consideration:

$$C = ((3\pi D_0 k^3)/(\lambda^3))\ [(\lambda^2 t^2 - 2\lambda t + 2)\ e^{\lambda t} - 2] \qquad \text{(equation 4)}^{59},$$

with *C* being the number of cells lysed in time *t*. To validate their model, they measured the abundance of a toxin, which is produced by lytically infected cells in known quantities, and compared the calculated number of lysed cells in dependence of the host cell density (Fig. 10)[59]. Although, the figures appear to coincide to a certain degree, it is notable, that the approach used in this study was unique and not further developed. In addition, the validation of the model by measuring the amount of produced toxin seems rather cumbersome and introduces unnecessary variance, which could be avoided by measuring the plaque radius over time and using equation 3.

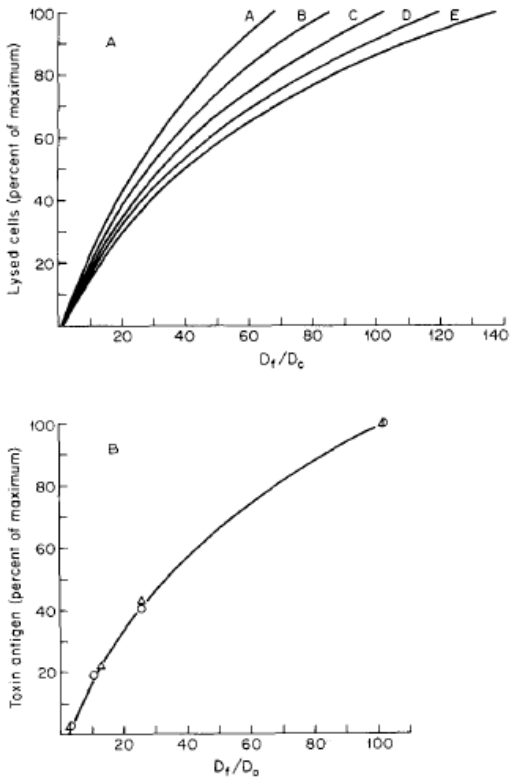

Figure 10. Equated values for the relative number of lysed cells per plaque for different host cell densities D_f (A), taken from[59]. Percentage of toxin antigen measured per plaque (corresponds to percentage of lysed cells) dependent on different host cell densities D_f[59] (B).

In contrast to all previous studies Yin used a particular phage in plaque assay experiments which showed no growth inhibition, even after several days[27]. Bacteriophage T7 was grown on its host, *E. coli*, and was only limited in growth by the availability of further surface (Fig .11)[27]. Notably, the figures presented are very different to all preceding graphs, in size of the plaques as well as in the time period of diameter augmentation (compare Fig. 9[24, 59] and Fig. 12[27]). Although, an explanation was not provided, at the time, Lee and Yin speculated in 1996, that the T7 phage might release by-products into the local environment, enabling host cells to sustain further plaque formation[60]. Yin proposes that the plaque is formed by an infection wave of a certain velocity, which is determined by an autocatalytic reaction-diffusion mechanism. The rate of plaque formation is therefore regulated by capacity of the phage to move and replicate in the agar layer. It is stated that diffusion alone

would not suffice to explain the kinetics of plaque formation, for several reasons: (i) diffusion would not sustain sharp boundaries, (ii) it would not result in linear growth dependence and (iii) it would not sufficiently explain the measured radial velocity. Yin hypothesizes that phage diffusion and phage replication are both responsible for plaque expansion and that phage particles are constantly arising at the propagating front[27].

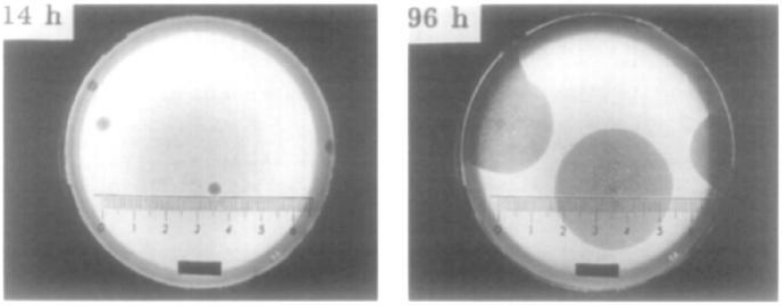

Figure 11. Images of bacteriophage T7 plaques at 14 and at 96 hours after infection, taken from[27].

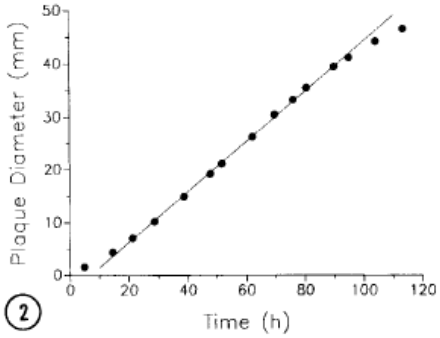

Figure 12. Bacteriophage T7 plaque diameters plotted against time after infection, taken from[27]. Plaques continuously increase in diameter linearly, even above 100 hours[27].

On basis of the previous observations a mathematical model was developed in 1992 by Yin and McCaskill[47] to investigate the relative impact of various aspects of plaque formation, including phage diffusion, phage adsorption rate, latent period, burst size and host cell growth. The approach is based on a model for RNA molecules in a capillary consisting of a coupled replication and diffusion reaction[61]. The model for plaque formation expresses the radial position and time dependence of the phage (virus), the host (bacterium) and the infected host and is summarised in a travelling-wave function describing the velocity of radial propagation. To obtain this model, interactions among the following three components were included: virus (*V*), bacterium (*B*) and infected host cell (*I*),

$$V + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} I \xrightarrow{k_2} \mathrm{Y} \cdot V, \qquad \text{(taken from[47])}$$

With k_1 being a constant for the rate of adsorption for the virus particle to its host, k_{-1} being a constant for the rate of desorption for the virus particle to its host, k_2 being a constant for the death rate (or lysis) of infected host cells and Y being the yield of new virus particles formed by one lysed host cell. Focusing in more detail on the various parameters influencing plaque propagation, the following insights were gained: the velocity scales with the square root of viral diffusivity, the velocity is dependent on the adsorption rate of the phage and this dependence has a maximum (as seen for experimental data, see p.13). It was further reasoned that k_{-1} can alter the rate in which the viral

adsorption equilibrium is obtained. With increasing k_{-1} an equilibrium between absorbed and unabsorbed phages is eventually reached and the velocity will be independent of k_{-1} (Fig. 13)[47].

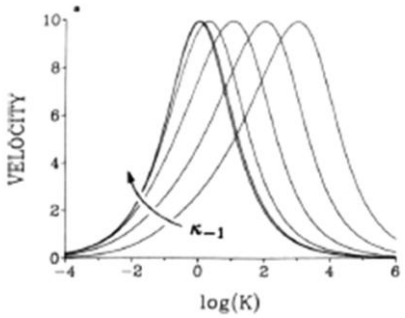

Figure 13. Direct relation between velocity and adsorption constant *k* as well as desorption rate k_{-1}, taken from[47]. The rate of adsorption is increased with increasing k_{-1}, albeit at constant *k*[47].

Considering *k* as a dimensionless equilibrium adsorption constant, it can be concluded that for small *k* the adsorption of the phage to its host is slow. If *k* is now increased either by increasing k_1 or the host cell density B_0, the consequential faster adsorption induces a higher velocity of plaque propagation than expected. On the contrary, it was observed that increasing an already large adsorption rate further, induces a lower velocity of plaque propagation. This is consistent with experimental data about the propagation velocity of plaques which were grown on different host cell densities. At high host concentrations plaques were found to grow more slowly. This experimental set-up can be used to gain insights on rates of adsorption, as there is an implicit relationship between the adsorption rate and host cell density (see p. 12). Yin speculated that under high host cell density conditions effective viral diffusivity might be disturbed, prohibiting further plaque enlargement. Under low host cell densities, diffusivity can occur without hindrance, allowing further plaque propagation. When comparing his equilibrated-adsorption model to the heuristic model developed by Koch[57], Yin concludes that Koch's model does not sufficiently account for the dependence of the velocity from the host cell density, since the conditions used in his model were moderate, not including values from extreme conditions (very high or very low host cell density)[47]. It is further shown that the equilibrated – adsorption model closely matches the experimentally obtained for bacteriophage T7[47], suggesting an important role of the adsorption rate for plaque propagation.

In conclusion the proposed model shows a monotonic increase of plaque size in time under low host cell density conditions (demonstrating that plaque growth depends on the adsorption rate), which is slowly compensated for and later inverted with increasing host cell densities, as phage particles become slowly unable to diffuse (demonstrating that plaque growth depends on diffusivity). Further effects that could influence the plaque propagation rate at high host cell concentrations are the changed metabolic state of the host or reversible high-multiplicity adsorption.

The validity of this model was further tested by You and Yin in 1999[32]. Restricting their number of *a priori* assumptions they show the existence of a travelling wave solution for reproducing viruses during plaque propagation, which was only presumed for the earlier model. They further demonstrate for a range of yields and adsorption rates the validity of a numerical model.

However, in 2002 a study was published by Fort and Méndez[62], contradicting the model proposed by Yin and colleagues[32]. The model was criticised in view of the fact that the time delay between virus

entry into the host cell and lysis of the host cell (latent period) was not taken into account. This was said to result in an inadequate representation of experimental data by the model, which can be abolished by including the time delay in the classical reaction-diffusion model (Fig. 14)[62]. Notably, also with this model, the plaque propagation velocity is still a function of the host cell concentration. The improved model by Fort and Méndez was further validated by an additional study by Ortega-Cejas *et al.*[63]. An analytical solution for the velocity of plaque propagation was found within distinct parameter values.

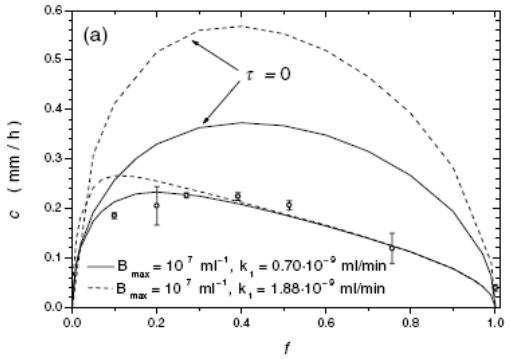

Figure 14. Bacteriophage T7 plaque propagation velocity *c* in dependence of relative bacterial concentration *f*, taken from[62]. Upper curves show propagation velocity estimates using classical model by Yin *et al*[47]., while lower curves show propagation velocity estimates using time-delay model suggested by Fort *et al.*[62]. Uncertainty of k_1 is taken into account by comparing two different values for k_1 (see inset)[62]. Experimental data (open circles) from Yin *et al.*[47] correspond to an initial bacterial concentration of 10^7 ml^{-1}. Velocity predictions for the time-delay model are compatible with experimental data[62].

All in all, despite several setbacks and uncertainties regarding the reasonability of attempting to model plaque propagation[62], it appears that a reasonable model for momentarily limited conditions can be used to model plaque growth rates.

7. Competition assays

Comparisons of the fitness status of different phages can be carried out in various scenarios. Fitness differences of the same phage albeit mutated can be seen within the same plaque as described before for the star mutant[31] or in the asymmetric plaques described by Lee and Yin for the T7 phage[38]. Fitness differences can also be determined by measuring the concentration of different phages in liquid culture over a distinct period of time, as presented earlier for the study by Bull *et al.* analysing adaptation limits of bacteriophages[39]. However, in this context it is not a direct competition of the phages rather than a comparison of their characteristics. This does not necessarily predict the outcome of competition assays. In order to conduct experiments which result in direct competition of different phages it is necessary to distinguish them. In an early study by Hershey[35] this was possible because a spontaneous mutation of the wild-type T-even phage resulted not only in a shortened latent period, but also in a plaque shape of the mutant which was distinguishable from the wild-type plaques. It was found that the wild-type phages out-grew the mutant phages, with the shorter latent period, in liquid culture assays. Growth rates of both phages were determined by

plating the filtered culture, containing both phage types on agar plates and determining their proportions over several transfers. Abedon *et al.* speculate that this might account for an adaptation of the T-even phage to low host density conditions[48]. In more recent studies on this topic different phages are usually genetically marked to allow distinction. A commonly used marker is the *lacZ* gene from *E.coli*, which is able to restore β-gal activity under IPTG and X-gal conditions in its functional version, but not in its inactive form. For competition assays, one phage strain is labelled with the functional *LacZ* marker (resulting in blue coloured plaque), while the other strain is labelled with the inactive marker (resulting in clear plaques)[8]. As described earlier (see p.13) Shao and Wang used this system to compete various isogenic phage λ strains that differed in their adsorption rate, to see which strain would display higher relative fitness[8]. The same technique was further utilised by Gallet *et al.* for competitive production–emigration transfer experiments[46]. For this purpose *LacZ* marked low adsorption wild-type (LA-wt) and high adsorption mutant (HA) strains were plated on appropriate host cells in different concentrations. Approximately 20 LA-wt plaques were opposed to 180 HA plaques. After overnight incubation liquid medium was poured on the plates and incubated for 30 minutes, before it was collected and used to initiate plaque growth on a fresh agar-plate. This procedure was repeated up to three times and was thought to simulate the spreading of emerging phages[46]. Notably, the LA-wt strain increased its relative frequency from initially 10 % to over 98 % in a single production-emigration step, displaying a selective advantage of the low adsorption strain in a non-liquid environment.

Competition of phages cannot only occur among host cells but also within one host cell. A bacterium can be infected with two or more phages at the same time. Interestingly, it was found that only one of the phages will be liberated after lysis of the host cell, referred to as the *mutual exclusion effect*[64]. The phage that completes its life cycle is partially dependent on the surrounding circumstances, e.g. possible time delay between infections with each phage, but as a general rule it was proposed that there are *strong* phages, which are more likely to dominate during a multiple infection, and *weak* phages, that do not. Exclusion was found to take place between related phages but also when phages were not related, suggesting a universally applicable rule[64]. However, it was shown that the phage which is not able to replicate, has an impact on the course of the infection, as it diminishes the yield of new phage particles produced by the host cell. This is referred to as the *depressor effect* [64]. The depressor effect does not occur when one bacterium is infected with several identical phages, in order to prevent self-interference.

In conclusion, it can be said that there are several approaches to investigate fitness advantages of phages in competitive assays. The advantage of competition assays lies in the fact that results obtained by these methods resemble more closely natural fitness gains or losses under distinct circumstances, as a direct comparison can be conducted.

Future directions and conclusions

The plaque assay system was first described for bacteriophages and bacteria. However, the improvement of tissue culture allowed using the plaque technique also for animal viruses on a monolayer of tissue cells. The principle of the assay is the same, with some exceptions. The host cells are not cultured within an agar layer, but on top or under a soft agar layer[29]. For some viruses the agar layer can be omitted completely and replaced by liquid medium[29]. The visibility of the plaques is

often improved by staining the live cells[65]. Studies on animal viruses gave additional insights and technical difficulties that arise with the plaque technique.

It was observed that plaques can appear at different time points after incubation[29]. This affects the sensitivity of the assay, since a long time delay before counting plaques (to include the maximum number of plaques) increases the likelihood of overlapping of early plaques[29]. In order to obtain reproducible and comparable data it is necessary to determine an optimal time after infection to count plaques[29]. This can be achieved by measuring the distribution of plaque sizes on a sample plate[29].

The time delay of plaque development seen for animal viruses is not found in bacteriophage plaque assays. A possible explanation for this discrepancy may be an observation made by You in 1999, stating that phages cannot initiate plaques on bacteria, that are in stationary growth[32], indicating that plaque appearance depends on that status of the cell. If this were also the case for eukaryotic cells, it could explain why this dependency affects animal virus plaque assays but not bacteriophage plaque assays, as the generation time of eukaryotic cells is significantly longer compared to bacteria.

This example demonstrates current technical issues of the plaque assay technique. Furthermore, a detailed comprehension of how host cells affect the plaque formation is still required. There could be a role for the metabolic state of the host cell, but lysed host cells could also release factors influencing the plaque development and adjacent host cells. Analysing the role of the host cell could lead to more precise determination of fitness differences of bacteriophages, or viruses in general.

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Appendices

Appendix I. Classification of bacterial viruses, taken from 4[4].

Order Family Genus	Description of order/family	Type species
	Double-stranded DNA viruses	
Caudovirales	tailed phages, genome linear dsDNA	
Myoviridae	contractile tails	
"T4-like viruses"		coliphage T4
"P1-like viruses"		coliphage P1
"P2-like viruses"		coliphage P2
"Mu-like viruses"		coliphage Mu
"SP01-like viruses"		Bacillus phage SP01
"ΦH-like viruses"[a]		Halobacterium phage ΦH
Siphoviridae	long, noncontractile tails	
"λ-like viruses"		coliphage λ
"T1-like viruses"		coliphage T1
"T5-like viruses"		coliphage T5
"L5-like viruses"		Mycobacterium phage L5
"c2-like viruses"		Lactococcus phage c2
"ψM1-like viruses"[a]		Methanobacterium phage ψM1
Podoviridae	short, noncontractile tails	
"T7-like viruses"		coliphage T7
"P22-like viruses"		enterobacteria phage P22
"ϕ29-like viruses"		Bacillus phage ϕ29
Tectiviridae	lipid-containing, double icosahedral capsids, produce tail-like tube upon nucleic acid ejection, genome linear dsDNA	
Tectivirus		polyvalent phage PRD1
Corticoviridae	lipid-containing, icosahedral capsid, genome circular dsDNA	
Corticovirus		Alteromonas phage PM2
Plasmaviridae	enveloped, pleomorphic, genome circular dsDNA	
Plasmavirus		Acholeplasma phage L2
Lipothrixviridae[a]	enveloped, rod-shaped, genome linear dsDNA	
Lipothrixvirus		Thermoproteus virus TTV1
Rudiviridae[a]	nonenveloped, rod-shaped, genome linear dsDNA	
Rudivirus		Thermoproteus virus TTV4
Fuselloviridae[a]	nonenveloped, lemon-shaped, genome circular dsDNA	
Fusellovirus		Sulfolobus virus SSV1
Unassigned Genus SNDV[a]	droplet-shaped, genome circular dsDNA	Sulfolobus virus SNDV

Appendix I (*continued*)[4]

Order Family Genus	Description of order/family	Type species
	Single-stranded DNA viruses	
Inoviridae	nonenveloped, filamentous or rod-shaped, genome circular ssDNA	
Inovirus		coliphage Ff[b]
Plectrovirus		Acholeplasma phage L51
Microviridae	nonenveloped, icosahedral, genome circular ssDNA	
Microvirus		coliphage ϕX174
Spiromicrovirus		Spiroplasma phage SpV4
Bdellomicrovirus		Bdellovibrio phage MAC1
Chlamydiamicro-virus		Chlamydia phage Chp1
	Double-stranded RNA viruses	
Cystoviridae	enveloped, icosahedral, genome segmented linear dsRNA	
Cystovirus		Pseudomonas phage ϕ6
	Positive-sense, single-stranded RNA viruses	
Leviviridae	nonenveloped, icosahedral, genome linear ssRNA	
Levivirus		enterobacteriophage MS2
Allolevirus		enterobacteriophage Qβ

[a] Infect *Archaea* spp.
[b] Collective designation for coliphages M13, f1, and fd