

# Persistence of bacteriophage T4 in a starved *Escherichia coli* culture: evidence for the presence of phage subpopulations

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Bacteriophage T4 is able to adjust its development to the growth parameters of the host cell. Here, we present evidence for the production of two different subpopulations of phage particles, which differ in their ability to infect starved *Escherichia coli* cells. The ability of phage T4 to produce a fraction of virions unable to infect starved cells is linked to the functions of genes *rI* and *rIII*, as well as *rIIA*. This may represent the adaptation of phage T4 in order to persist in unfavourable environmental conditions.

## INTRODUCTION

Bacteriophage T4 is able to adjust its development to the physiological state of the host cell (Hadas *et al.*, 1997). This adjustment depends not only on the ability of the bacterial cell to produce progeny phage particles and lysis proteins but also on other factors and processes (Łoś *et al.*, 2003). In particular, the development of bacteriophage T4 is generally longer than the time necessary to form a sufficient number of progeny phages. Interestingly, under conditions of a very slow bacterial growth, T4 development may even be stopped (Łoś *et al.*, 2003). It also stops at the early phases of development if the bacterial host is not growing at all (Kutter *et al.*, 1994). This mimics, to some extent, lysogenization, which may be accomplished by temperate bacteriophages. Lysogenization is a strategy that was adopted by some groups of bacteriophages. It allows them to cause their genetic material to be stably maintained by a host cell, instead of undergoing a lytic cycle. This stage is maintained until changes in the host's environment provoke the phage to switch its development to the lytic mode. Such alternative (lytic and lysogenic) life cycles occur only in temperate phages. However, some virulent phages can mimic this adaptation; this can be observed in phage T4. When host bacteria are starved, phage T4 does not form progeny virions. Instead, its genome can be maintained in the infected host cell until the host's growth resumes, and then a small burst of progeny phage is produced and the cell is killed by lysis. (Kutter *et al.*, 1994). This maintenance of the phage genome in the cell, without its replication, is called pseudolysogeny.

It is obvious that, owing to the altered biology of starved cells relative to rapidly growing bacteria, bacteriophages had to develop mechanisms allowing them to cope with such conditions. Starved *Escherichia coli* cells have multiple adaptations. For instance, starvation reduces the abundance and activity of the *E. coli* protein biosynthesis apparatus, which allows for a reduction in the energetic costs of life. As a result, the transcription pattern changes, mainly due to the use of different sigma factors by the RNA polymerase core (Hengge-Aronis, 2002).

Although starvation is common for natural bacterial populations, so-called laboratory conditions, which usually provide the best possible conditions for bacterial growth, were routinely used for investigations of bacteriophages' development in their bacterial hosts. This might lead to conclusions that should not be generalized to naturally occurring bacterial populations, which co-exist with bacteriophages. Therefore, to fill the gap in our knowledge about phage–host interactions, we decided to study infections of starved *E. coli* cells by phage T4 and its *rI*, *rII* and *rIII* mutants, which previously showed altered development in slowly growing cells (Łoś *et al.*, 2003).

## RESULTS

Diluted Luria–Bertani (LB) medium was inoculated with an overnight culture of *E. coli* MG1655. The amount of nutrients present in the medium allowed for bacterial growth until the OD<sub>600</sub> of the bacterial culture reached a value of approximately 0.3. Then, bacterial growth stopped due to

nutrient depletion, and thus the starvation began. After 1 day of starvation, the bacterial cultures were infected with bacteriophage. The number of c.f.u., infective centres, free phage in supernatant and infected bacteria were monitored for a long period (50 days). Two m.o.i. were used: 1 and 0.01.

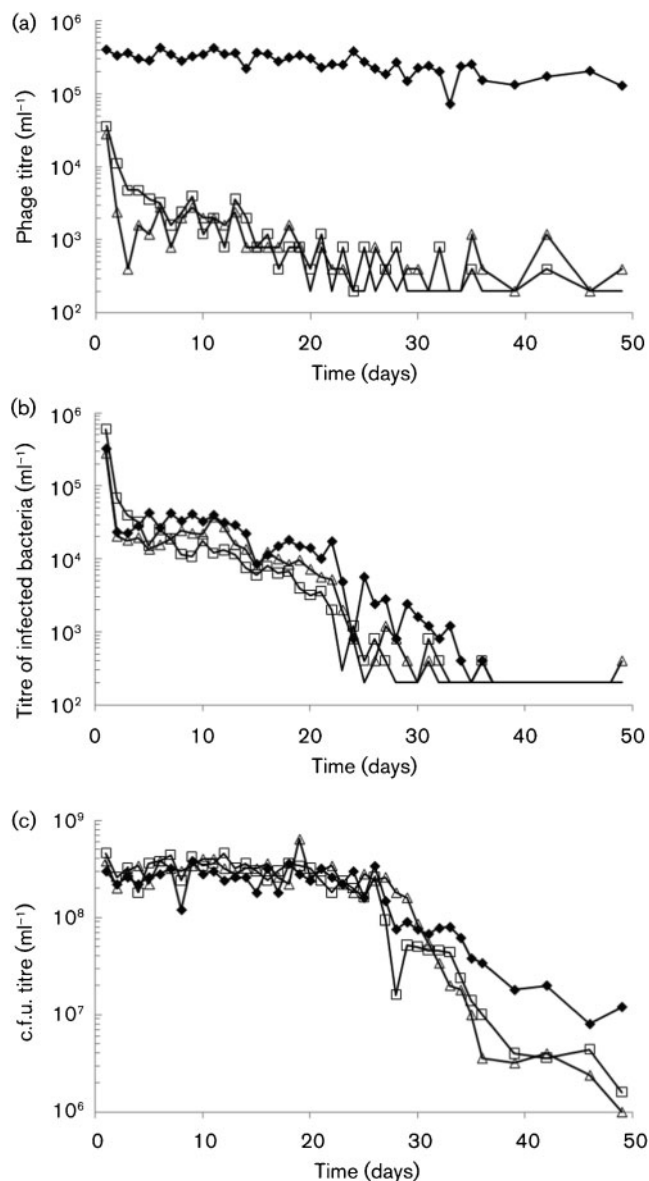
The results were similar for both m.o.i. used. In cultures infected with phages T4rI, T4rII and T4rIII, the number of free phage gradually decreased to the limit of detection of the

titration method used (Fig. 1). This was not the case in cultures infected by phage T4D, where the titre of free phage was stable during the course of the experiment (Fig. 1).

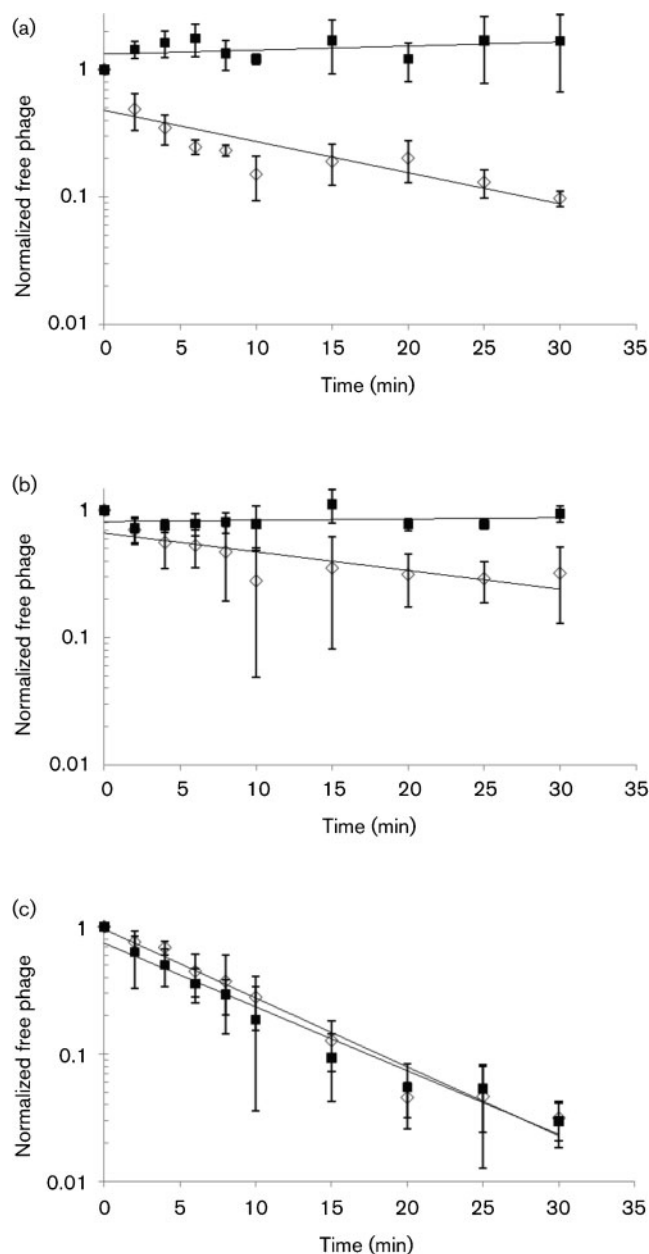
During all the experiments with starved cultures, the bacterial titre decreased over time. However, the fall in the number of bacterial cells was relatively slow, in contrast to the rapid decay of the number of infective centres in the same cultures. Nevertheless, differences in the course of infections caused by mutant phages, T4rI, T4rII and T4rIII, and the wild-type phage, T4D, were observed. Although the titre of live bacteria and the titre of infected bacteria were approximately the same in experiments where all three mutant phages were tested, the number of free, unadsorbed T4D phages was higher. Importantly, the number of wild-type phages was relatively stable during the whole experiment. The number of infected bacterial cells, which was measured by washing away unadsorbed phage particles and titrating the remaining bacterial cells on a bacterial lawn, was similar in all experiments. Thus, the relatively high number of unadsorbed T4D phages showed no influence on the number of infected bacterial cells. This experiment was repeated several times using different lysates and different m.o.i. Although we observed that the amount of phage T4D stabilized at different levels in different experiments, it was always at least tenfold greater than those of the mutant phages tested.

The above-described results suggested that a fraction of wild-type bacteriophage may lose their affinity for their bacterial hosts during the infection of starved cells. This might be caused by either a difference in the bacterial cells or a difference in phage affinity. Although the former option seems to be more reasonable, the course of the experiment with mutant phages showed that they adsorbed relatively efficiently (Fig. 1a).

To test whether the observed results were caused by a lack of adsorption of phages in a starved culture, we performed an experiment which allowed us to compare the adsorption rate (on to starved and growing bacterial cells) of the wild-type phage T4D, with analogous phage lysates passaged through the starved bacterial culture. We found dramatic differences in adsorption rates of phages from these two groups (Fig. 2). When bacterial cells from a culture starved for 2 weeks at 30 °C were used, T4D phage that were not passaged through a starved culture adsorbed efficiently, in contrast to those extracted from the starved culture, which did not adsorb at all (Fig. 2a). During the analysis of adsorption of T4D phage that were not passaged through the starved culture, we observed that the drafted curve did not fit an exponential trend line. It seems likely that the adsorption proceeded during the first 10 min, and then slowed down considerably (Fig. 2a). This might be a manifestation of the presence of a non-adsorbing phage subpopulation, which remained in the culture after the faster-binding phage were titrated out. When adsorption experiments were performed at 37 °C, on bacteria starved at 37 °C, phages passaged through the starved culture

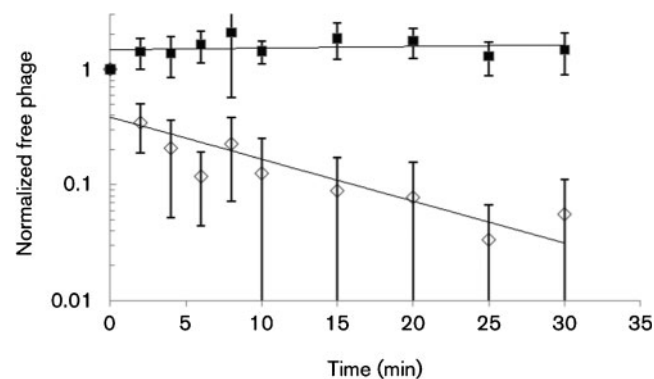


**Fig. 1.** Infection of starved cultures of *E. coli* MG1655 by bacteriophages T4D, T4rI, T4rIIA and T4rIII with an m.o.i. of 0.01. (a) Changes in phage titre. (b) Titre of infected bacteria. (c) Titre of c.f.u. Cultures infected by: ◆, T4D; □, T4rI (r48); ○, T4rIIA (r59); △, T4rIII (r67). Assay sensitivity was 400 phage ml<sup>-1</sup>; thus all points with a negative result were removed from the graph, and the baseline was set to 200.



**Fig. 2.** Adsorption of normal T4D phage lysate ( $\diamond$ ) and phage lysate passed through starved culture ( $\blacksquare$ ) using the following bacterial cultures: (a) starved at 30 °C, (b) starved at 37 °C and (c) exponentially growing at 30 °C. Values presented are the means from three independent experiments and SD are shown.

showed virtually no binding. We also saw that standard phages showed less efficient binding than that seen in cultures starved at 30 °C (Fig. 2b). The adsorption of phage from this lysate was efficient when it was performed at 30 °C in a culture of growing bacterial cells (Fig. 2c). This set of experiments indicated that the inability of a phage subpopulation to adsorb on to host cells is restricted to starved cultures only, and that binding to exponentially grown cells is not impaired.

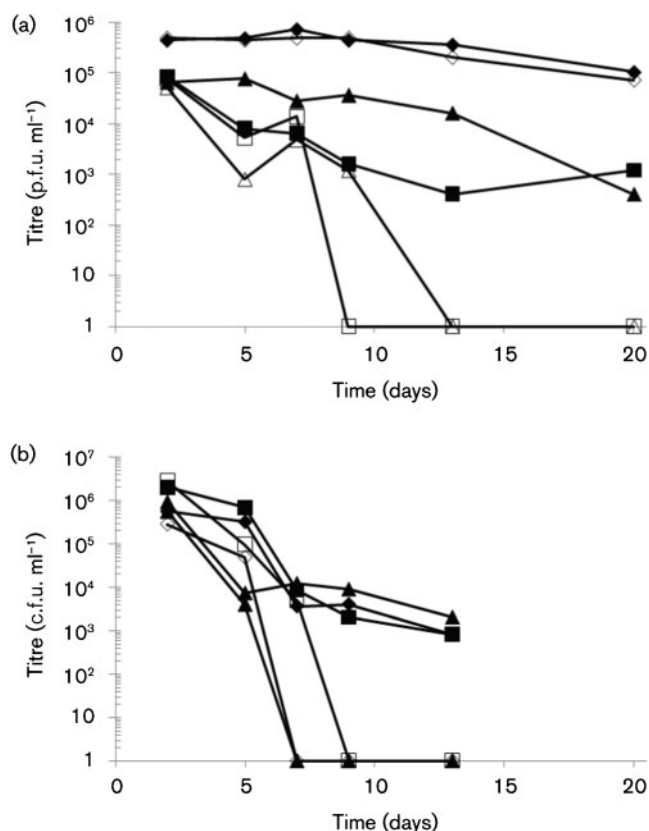


**Fig. 3.** Adsorption of regular ( $\diamond$ ) and passed ( $\blacksquare$ ) phages on exponentially growing cells suspended in metabolized media from 2-week-old bacterial culture (15× diluted LB).

Further experiments (Fig. 3) showed that the inability of phages passed through a starved culture to adsorb to bacterial cells was linked to the medium itself or to compounds released into the medium by bacteria, rather than to the bacterial cells themselves. When exponentially growing cells were resuspended in metabolized media, phages were unable to attach to the cells (Fig. 3).

To check whether the presence of phages with reduced binding affinity resulted from selection by adsorption of phages which adsorbed well to starved cells at 30 °C or by *de novo* production of daughter virions in starved culture, we performed an experiment in which *de novo* synthesis was blocked by the use of chloramphenicol. During the course of this experiment, all of the bacteria died before the end of the first week, which was observed as a lack of c.f.u. (data not shown). The changes in bacteriophage titres were virtually the same as in the previous experiments; the levels of phages T4rI and T4rIII were gradually decreasing and the level of phage T4D was stable after an initial drop (Fig. 4). An interesting conclusion drawn from this experiment is that titres of rI and rIII mutants in cultures poisoned by chloramphenicol showed a considerably faster rate of decay of phage titre, which may suggest that there was some phage production in the starved culture. However, the mean burst size of such a development would be  $<1$ . This suggestion is also supported by slightly (but reproducibly) higher titres of rI and rIII mutant phages (but not wild-type phage) when comparing chloroformed versus unchloroformed cultures (data not shown). Thus, the procedure of passing the T4D phage lysate through a starved culture allowed for an enrichment of a fraction of non-binding phages and the elimination of phages which bind to starved cells. Electron microscopic observations showed no differences in capsid morphology between phages from a standard lysate and a lysate which was passed through the starved culture (data not shown).

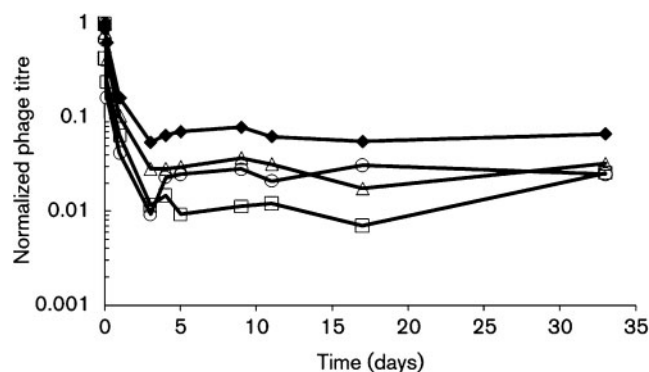
To test for a possible effect of the medium itself on the different phages used in this study, we monitored the



**Fig. 4.** Infection of starved cultures (closed symbols) and starved cultures supplemented with 100 µg ml<sup>-1</sup> of chloramphenicol (open symbols) of *E. coli* MG1655 by bacteriophages T4D (diamonds), T4rI (r48) (squares) and T4rIII (r67) (triangles). (a) Titre of phages present in the supernatant and (b) infective centres present in the cell pellet.

long-term exposure of phages T4D, T4rI, T4rII and T4rIII to metabolized medium, which was sterilized by filtration. The metabolized medium partially inactivated all phage stocks, but the inactivation occurred mostly during the first day of exposure. All phages showed similar susceptibility to inactivation. In all cases, the phage titre stabilized at the fourth day of the experiment, and remained stable for the next month (Fig. 5).

Owing to the fact that different phage subpopulations appeared during phage stock preparations, not during incubation with starved cells, we decided to check whether the lysis inhibition (LIN) mechanism (Rutberg & Rutberg, 1965) is involved in this process. To address this issue we performed an experiment whereby phage lysates were produced by infecting bacterial cultures grown under standard laboratory conditions (LB medium, 37 °C) with an m.o.i. <0.5, and after 25 min chloroform was added and the infected cells were lysed to release the progeny phages. To avoid cross-contamination of phage lysates with introduced phage stocks, which were composed of both phage types, we performed phage stock production by



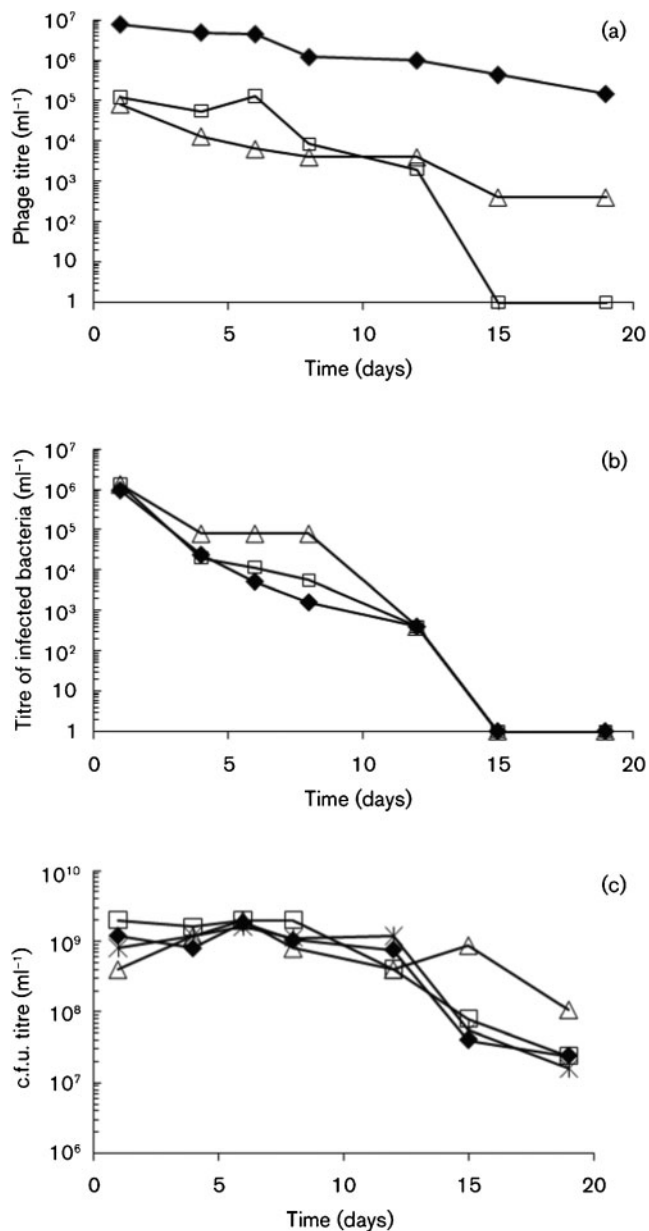
**Fig. 5.** Long-term exposure of various phage mutants to metabolized medium removed from 2-week-old culture. Normalized titres of T4D (◆), T4rI (r48) (□), T4rIIA (r59) (○) and T4rIII (r67) (△) are shown. Values presented are the means from three independent experiments.

repeating the process four times, each time using the phage lysate from the previous, interrupted phage development cycle. The phage lysates obtained at the end of this process should be virtually free of phages from the original phage stock, which might remain unadsorbed when the bacterial culture infection was performed. When this type of lysate, free from phage particles produced in lysis-inhibited cells, was used to infect starved bacterial cultures, we obtained results very similar to these given by regular lysates (Fig. 6).

## DISCUSSION

Infections of starved bacterial cultures by bacteriophage T4 mimics, to some extent, the situation that is the most challenging one for any organism – to persist in its habitat under non-permissive conditions. Success in this task brings the possibility of colonizing other niches, especially if they are spatially separated. In the case of temperate phages, such a task can easily be accomplished by lysogenization of host cells. As long as the host cell is able to persist under unfavourable environmental conditions, a prophage in the bacterial chromosome is safe.

Virulent phages did not develop such an adaptation. However, they also have to persist under unfavourable conditions in order to survive. It was presented previously that one of members of this group, phage T4, was able to react to different growth rates of host cells by modifying its development rate. This ability was, in turn, dependent on the presence of functional genes involved in the LIN response (Łoś *et al.*, 2003). The ability to trigger LIN only in reaction to superinfection by other T-even phages serves as an advantage only in an environment where the number of available hosts is smaller than the number of bacteriophages, and both populations are not dispersed. However, the presence of an effective system preventing premature lysis may be used in many ways for the benefit of the phage. In the case of bacteriophage T4, the benefits postulated so



**Fig. 6.** Infection of starved cultures of *E. coli* MG1655 by bacteriophages T4D, T4rI and T4rIII with an m.o.i. of 0.01 from LIN-free lysates. Changes of phage titre (a), titre of infected bacteria (b) and titre of c.f.u. (c) in cultures infected by T4D (◆), T4rI (□), and T4rIII (△). C.f.u. level in an uninfected culture is shown in panel (c) (\*).

far are: increasing burst size and the length of the development cycle in response to superinfection (Abeldon, 1992) and controlling bacterial growth-rate-dependent cycle time and allowing for stable pseudolysogenic interactions with the host cell (Łoś *et al.*, 2003). In this study we showed how the presence of functional LIN genes influences phage persistence under conditions that disable normal phage development.

Following infection of bacteria that are in the stationary phase of growth, bacteriophage T4 is able to produce some phage enzymes, but replication of phage DNA is halted. This occurs at a relatively short time after infection (Kutter *et al.*, 1994). During the long-term infection of starved bacterial cells investigated in this study, we observed that the titre of infected bacterial cells fell constantly and eventually reached the limit of detection. This decrease in the number of infected cells was quicker than the decrease in the number of c.f.u. Thus, the presence of resident bacteriophages may cause the bacterial cell to be more prone to death under such conditions, even without evident phage progeny release. We assume that it would be very unprofitable for phage to infect starved bacterial cells if bacterial growth is not restored relatively quickly. The most intriguing observation was the almost constant titre of free bacteriophage T4D virions during the experiment. This could be explained in several ways. One possibility was *de novo* production of progeny phages only after infection with T4D phage. However, this was excluded in the experiment where *de novo* synthesis of protein was blocked by the addition of chloramphenicol. Since addition of this inhibitor of protein synthesis did not change the persistence of bacteriophage T4D in starved culture, the conclusion can be drawn that the observed phages are not produced *de novo*, but rather that they were introduced to the bacterial culture while being infected with the phage stock.

We propose that the phage population introduced to the culture consisted of two populations: (i) phages that can infect starved cells and (ii) those which are not able to do so. The presence of two different subpopulations had already been demonstrated by a dual sedimentation of T-even phage lysates (Cummings *et al.*, 1969), but it is not clear whether the same kinds of subpopulations were observed in this case. Definitely, phage T4 has a mechanism allowing it to prevent adsorption on to host cells by retracting its tail fibres. This may be a reaction to unfavourable environmental conditions, such as low pH, low ionic strength or low temperature (Conley & Wood, 1975).

Another intriguing question is why no such subpopulations are observed for phage T4 rI, rII and rIII mutants. Since bacteriophage T4 morphogenesis occurs on the cell membrane (Leiman *et al.*, 2003) and this is also the place where RI, RIIA and RIII proteins are expected to function during phage T4 development, one may suggest a role for these proteins in the morphogenesis of phage particles with altered affinity to bacterial cells. Another explanation might be their involvement in the timing of lysis. During the preparation of T4D phage lysates, LIN occurs (Abeldon, 1992). This phenomenon prolongs phage development significantly, allowing phage to produce significantly more progeny virions. However, it was demonstrated in this work that phage particles of altered binding affinity are not only produced in lysis-inhibited cells. Phage lysates made by premature lysis of infected bacteria, before LIN can be

triggered, also proved to contain a fraction of phages which do not bind to starved bacterial cells. Thus, an inability to switch on the LIN mode is not the reason why T4rI, T4rII and T4rIII mutants do not produce such virions. On the other hand, it is likely that proteins involved in LIN are also involved in different activities. This was demonstrated for the T4rII mutant, which also produces two different subpopulations of virions. Moreover, the rapid lysis phenotype of rII mutants is not directly linked to premature LIN termination, but rather to premature lysis of *E. coli* B. On *E. coli* K-12, T4rII mutants show wild-type growth and plaque properties under standard laboratory conditions of bacterial growth (Paddison *et al.*, 1998). Thus, the progeny phages of rII mutants were, contrary to rI and rIII mutants, produced in cells which could undergo LIN. However, similarly to rI mutants, phages with rII (but not rIII) deficiency show altered specificity of DNA packaging in *E. coli* B (Carlson & Kozinski, 1974), and thus, RI and RII may be suspected to play a role in virion morphogenesis.

Production of phages with lowered affinity to bacterial cells, or phages which can bind only to growing bacterial cells, seems to be an effective strategy for phages which are constantly endangered by adsorption to cells which are starving or are suspected to be excreted outside of the relatively safe environment of the mammalian gut. In our experiments, we observed the relatively rapid loss of the ability to form infective centres by infected bacterial cells, which was also faster than the decay in the number of c.f.u. This suggests that bacteria that are infected by phages that enter a pseudolysogenic state are less vital; thus their ability to give a burst of activity after the addition of nutrients drops constantly as starvation is prolonged. Such a situation may be caused by incomplete halting of phage development in response to the host's starvation. However, even if this leads to the lysis of bacterial cells by phage-encoded lytic systems or to killing the cells by activation of other phage genes, the virus is most probably unable to produce progeny, or progeny production is severely impaired, giving a mean burst size  $<1$ . The latter possibility was suggested by a significantly faster drop in the titre of T4rI and T4rIII phages in chloramphenicol-treated cultures than in a starved culture without chloramphenicol (Fig. 3a). Hence, for the phage, it is very unprofitable to infect starved bacterial cells since the capsids of phage T4 are more stable than infected bacterial cells. Therefore, the strategy of producing two types of virions with altered affinity to bacterial cells may provide an effective adaptation to an unfriendly environment, which does not compromise the ability to overtake populations which are growing rapidly.

Based on our results, we propose that, during the development of phage T4, two subpopulations are formed. They are indistinguishable by electron microscopy, but they show altered affinities to host cells which are starved. Different modes of development of phages T4D and T4rI in slowly growing cultures at low temperature (25 °C) have

already been reported (Łoś *et al.*, 2003). It is also possible that in the experiments presented in that paper the observed results were caused by a lack of adsorption of a major fraction of T4D bacteriophages, while T4rI retained their abilities to adsorb and develop in bacterial cells. However, no development of T4D was detected, which may suggest that in the T4D lysate there was no fraction of phages capable of binding and development under the given conditions. Alternatively, development might not be intensive enough to influence the observed phage titre. On the other hand, the presence of bacterial cells infected by T4D in these experiments clearly shows that this phage produces mixed populations. Regardless of the lack of ability of phage T4 with mutations in rI, rII or rIII genes to produce two subpopulations, this feature appears not to be directly linked to LIN; it is triggered during phage development, as our attempts to produce a uniform phage population by not allowing LIN to occur failed.

## METHODS

**Bacteria and phage strains.** *E. coli* MG1655 strain (Jensen, 1993) was used for all experiments. Bacteriophage T4D, and its isogenic mutants T4rI (r48) (Doermann & Hill, 1953), T4rIII (r67) (Edgar *et al.*, 1962) and T4rIIA (r59) (Benz & Berger, 1973) were employed.

**Growth conditions.** Long-term experiments were performed using 15× diluted LB medium (Sambrook *et al.*, 1989) with the NaCl concentration adjusted to 1% (the level which is present in the standard LB medium). Diluted LB supported cell growth to OD<sub>600</sub>~0.3, which limited the probability of anaerobic growth at the later stages of exponential growth and also lowered the accumulation of various metabolites owing to the lower amount of nutrients and higher oxygen availability during growth. Bacteria were infected after 24 h incubation in the indicated media, which allowed them to enter into the stationary phase of growth. In experiments where chloramphenicol was added the culture was prepared as indicated above, but after 24 h the culture was split into two flasks. Chloramphenicol was added to one of the flasks to a final concentration 100 µg ml<sup>-1</sup>, and both cultures were incubated for an additional 3 h prior to addition of phage. Overnight cultures were prepared in standard LB broth. All long-term experiments were performed in shaking flasks at the indicated temperature. Prolonged incubation of bacteria in 15× diluted LB medium without chloramphenicol resulted in minor pH variations which were in the range of ±0.2 pH units (data not shown).

### Phage lysate preparation.

**Regular lysate.** Regular phage lysate was prepared as follows. LB broth was inoculated with an overnight bacterial culture. When the OD<sub>600</sub> of the culture reached 0.1, phage lysate was added to an m.o.i. of 0.001. Following culture lysis, a sample was centrifuged at 2500 g and phage lysate was harvested.

**LIN-free lysate.** LIN-free lysate was prepared as follows. LB broth was inoculated with an overnight bacterial culture. When the OD<sub>600</sub> of the culture reached 0.1, phage lysate was added to an m.o.i. of 0.7. After 25 min, chloroform was added (20% of culture volume) and the culture was vortexed, centrifuged and phage lysate was harvested.

**Passaged lysate.** For preparation of a passaged lysate, we inoculated 15× diluted LB (with NaCl concentration adjusted to 1%) with an overnight culture. After 24 h of incubation at the indicated temperature, phage lysate was added to the culture to a final m.o.i. of 0.5. The culture

was incubated at the indicated temperature for 2 weeks. Then, bacterial cells were centrifuged 2500 g and bacteriophages present in the supernatant were used in subsequent experiments.

**Phage and bacteria titration.** Phage titration was performed in disposable plastic Petri dishes (90 mm diameter; Merck) containing 25 ml of bottom LB agar (1.5 %). Three millilitres of top agar (0.7 % Bacto-agar, 1 % Bacto-tryptone, 0.5 % NaCl), containing 0.2 ml of an overnight bacterial culture were poured on to the plate. For estimation of the unadsorbed phage titre, 1 ml of the bacterial culture was centrifuged at 2000 g for 15 min. The supernatant was used for preparing serial dilutions for phage titration on double-agar-layer plates. The pellet was washed three times in TM buffer (10 mM Tris pH 7.4, 10 mM MgSO<sub>4</sub>), resuspended and serial dilutions in TM buffer were made. Then, serial dilutions were titrated on both Luria agar plates, to estimate the c.f.u. titre, and on double-agar plates, to estimate the number of infected bacteria.

Plates were incubated at 37 °C for 16 h. To estimate the total number of phages, 200 µl of bacterial culture was mixed with an equal amount of chloroform and vortexed vigorously for at least 30 s in order to lyse phage-infected cells. After centrifugation, the supernatant was used for preparing serial dilutions in TM buffer and titrated on double-agar plates. Plates were incubated at 37 °C for 16 h.

**Phage adsorption experiments.** Phage adsorption experiments were performed in test tubes filled with an appropriate bacterial culture. Test tubes were incubated at the indicated temperature in a thermoblock. Phage lysate (or a dilution thereof) was added at time '0', and the culture was vortexed. Samples (50 µl) were withdrawn at the indicated time points and centrifuged at maximal speed (8000 r.p.m.) in a microcentrifuge (Eppendorf mini spin plus) for 1 min. 10 µl of the supernatant were aspirated and diluted immediately in 990 µl of TM buffer. Phage load in samples was estimated using spot titration (Sambrook *et al.*, 1989).

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