Received Date : 08-Oct-2013

Revised Date : 01-Jan-2014

Accepted Date : 01-Jan-2014

Article type : Research Letter

Editor : Richard Calendar

Bacteriophage T4 can produce progeny virions in extremely slowly growing *Escherichia coli* host: comparison of a mathematical model with the experimental data

Running title: **Host growth rate and T4 phage development**

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Keywords: bacterial growth rate, adsorption rate, development of T4 phage, chemostat cultures

Abstract

Development of bacteriophage T4 depends on the physiological state of its host cell. Based on previous studies performed under laboratory conditions with different media determining various growth rates of *Escherichia coli*, a mathematical model was developed which suggested that phage T4 development cannot proceed efficiently in bacteria growing with a doubling time longer than 160 min. Contrary to this prediction, using a chemostat culture system allowing for culturing *E. coli* at different growth rates without changes in the medium composition, we found that T4 can yield progeny in host cells growing with a doubling time as long as 21 h. Our results indicate that the actual limiting growth rate of the host culture for development of phage T4 is about 0.033 h^{-1}, corresponding to the doubling time of about 21 h.

Introduction

Bacteriophages are the most abundant and highly dynamic group of organisms in the biosphere (Fuhrman, 1999). The total number of phages on Earth is estimated at about 10^{31} particles and is ten times higher than the predicted number of bacterial cells (Børsheim, 1993; Clokie *et al*., 2011; Hatfull & Hendrix, 2011). Worldwide phage infections are calculated to be approximately 10^{23} per second, which makes them a fundamental regulating factor of the number of microorganisms - the producers of about 90% of biomass (Wommack & Colwell 2000; Suttle, 2007). Phages are also considered as natural weapons against pathogenic bacteria (Pirnay *et al*., 2011). Their antibacterial properties are used in phage therapy – against

antibiotic-resistant bacteria in humans (Takemura-Uchiyama *et al*., 2013) and animals (Kropinski *et al*., 2012), to combat bacterial infections in fungi and plants (Adriaenssens *et al*., 2012; Sajben-Nagy *et al*., 2012), and in food industry as antibacterial components of forage (García *et al*., 2008). Furthermore, phages are used in phage display technique (Sidhu, 2000, Golec *et al*., 2012), and as potential therapeutic gene-delivery vehicles or as vehicles for the delivery of vaccines (Clarc *et al*., 2004; Tao *et al*., 2013). On the other hand, their antibacterial properties cause substantial financial losses in the biofermentation industry (Marcó *et al*., 2012).

Phages can interact with bacteria in various ways. An explanation of these interactions is essential for understanding the influence of phages on the microbial world (Wommack & Colwell 2000; Weinbauer, 2004; Brüssow, 2013). Generally, the development of phages in bacteria depends on the physiological status and the number of bacterial cells (Hadas *et al*., 1997, Abedon *et al*., 2001, You *et al*., 2002). After infection, phages can initiate various developmental mechanisms: e.g. lytic or lysogenic cycle, pseudolysogeny, lysis inhibition. Lytic bacteriophage T4, on which we focused in this study, is able to control its development in response to different states of its host, *Escherichia coli* (Hadas *et al*., 1997). Under standard laboratory conditions (a high-density, fast-growing bacterial culture in a rich medium at 37°C with aeration),T4 appears to use a short latent-period (SLP) strategy which results in releasing of about 150 – 200 progeny particles in 25 minutes (Abedon *et al*., 2003). However, optimal laboratory conditions are far different from conditions encountered by T4 in its natural habitat. Optimal laboratory growth conditions may either disguise natural developmental mechanisms of T4, hindering their identification, or simply prevent the phage from initiating them as no selection is present to employ them.

Under conditions unfavorable for bacterial growth, T4 is able to initiate complex mechanisms enabling its persistence in the natural environment: pseudolysogeny (Łoś & Węgrzyn, 2012, Cenens *et al*., 2013), lysis inhibition (LIN) (Dressman & Drake, 1999; Golec *et al*., 2010) or prolongation of its development when bacterial growth rate (refered to as μ) increases (Rabinovitch *et al*., 2002, Golec *et al*., 2013).

The duration of phage T4 latent period depends on the bacterial μ (Abedon *et al*., 2001; Hadas *et al*., 1997; Rabinovitch *et al*., 1999; Rabinovitch *et al*., 2002; Golec *et al*., 2013). It was revealed that with decreasing μ, the rate of phage T4 release and burst size decrease while the eclipse and latent periods increase (Hadas *et al*., 1997; Rabinovitch *et al*., 2002). Based on results of one-step growth experiments in which different media were used to control the bacterial growth rate, Rabinovitch *et al*. (2002) presented a mathematical model of development of T4 in slowly growing *E.coli*. The model suggested that T4 develops in *E. coli* cultures with doubling times of about 140 minutes, and led Rabinovitch *et al.* (2002*)* to ask if any bacterium can release phages when its doubling time is longer than 160 min. Results of our recent experiments on development of T4 in slowly growing *E. coli* indicated that T4 produces progeny particles in bacterial cultures with doubling times significantly longer than 160 minutes (Golec *et al*., 2013). This led us to ask: what is the actual lowest growth rate of *E. coli* allowing for the development of bacteriophage T4? In this report, we present data from experiments carried out in a chemostat system that enabled us to study development of T4 in slowly growing bacteria in detail. We show that predictions based on the mathematical model of Rabinovitch *et al*. (2002) differ from results obtained in the experimental approach.

Materials and Methods

Bacterial and phage growth conditions

E. coli MG1655 strain (Jensen, 1993) and bacteriophage T4wt (our collection) were used in all experiments. Bacterial cultures for phage titration were grown overnight in laboratory flasks in LB medium (Sambrook *et al.*, 1989). Bacterial cultures used in experiments of adsorption and development kinetics of phage T4 were grown in phosphate-buffered (FB) minimal medium containing glucose (10 g l^{-1}), with stirring at 37°C in chemostats, as described previously (Golec *et al.*, 2013). The dilution rates (equal to the growth rates, μ) used were: 0.3, 0.2, 0.1, 0.05 and 0.033 h^{-1}, being equivalent to the generation time of: 2.5, 3.5, 7, 14 and 21 h, respectively.

Titration of bacteriophage T4

The number of bacteriophages (plaque-forming units, PFUs) were determined by a standard plaque technique. Briefly, double-layer LB agar plastic Petri dishes (diameter 90 mm) were used. LB agar (Sambrook *et al.*, 1989) was used as a solid medium (1.5% agar in regular plates and 0.7% agar in "top agar"). Twenty five ml of LB agar and 4 ml of "top agar" with 200 µl of an overnight bacterial culture were used to prepare double-layer LB agar Petri dishes. Serial dilutions of samples in TM buffer (10 mM Tris-HCl pH 7.2, 10 mM $MgSO_4$) were prepared. Two and half µl of serial dilutions were spotted onto a bacterial lawn. Plates were incubated at 37°C and plaques were counted after 16 hours.

Efficiency of phage adsorption

One ml of the bacterial culture from the stabilized chemostat culture was transferred to a 1.5 ml Eppendorf tube. Bacteriophages were added to *E. coli* cells to

multiplicity of infection (m.o.i.) of 0.1 and the mixture was incubated at 37°C. Samples of 100 µl volume were withdrawn at the indicated times, centrifuged at 4500 x *g* for 1 min at room temperature (RT) in a microcentrifuge, and the supernatant was titrated. Initial number of phages (100% of the used phages) was calculated by adding appropriate volume of the T4 lysate to a medium without bacteria, followed by titration. The number of adsorbed phages was determined as a decrease in PFUs in supernatant relative to the initial number of phages.

Kinetics of phage development in the chemostat

Five ml of the bacterial culture from the stabilized chemostat culture were infected with T4wt phage at the m.o.i. of 5. After 1 min of incubation at 37°C, free phage particles were removed by a washing procedure, repeated three times (centrifugation at 4500 x *g* for 1 min at room temperature (RT), resuspension in FB medium pre-warmed to 37°C). Then, the infected bacteria were added to the chemostat culture. PFUs (300 µl of samples treated with 300 µl of chloroform) were estimated 10, 15 and 20 min following the infection and then every 10 min for 300 min. The samples for estimation of the number of infection centers (ICs) (samples untreated with chloroform) were collected 10, 15 and 20 min after the infection. The real number of ICs was calculated by subtracting the number of plaques that were formed by free phages from the total number of plaques of all phages (i.e. phages present inside and outside of the bacterial cells).

Results

Kinetics of phage T4 adsorption on slowly growing bacteria

Adsorption is the first step in a life cycle of bacteriophages and was previously shown to depend on a number of environmental factors, such as ion concentrations,

organic cofactors' concentration, pH value and temperature (Storms *et al.*, 2010). In this study we analyzed kinetics of adsorption of phage T4 to slowly growing *E. coli* (Fig. 1). We used *E. coli* cultured in a chemostat system where the growth rate of bacteria was the only variable factor. We found no significant differences in the efficiency of phage adsorption on cells growing at μ ranging from 0.3 to 0.033. Therefore, we conclude that the host growth rate has no considerable influence on T4 adsorption in chemostat cultures.

Kinetics of development of phage T4 in slowly growing bacteria

Chemostat cultures of *E. coli* were used to examine the differences in kinetics of development of phage T4 in response to different growth rates of bacteria. We determined latent periods and burst sizes of T4 at five different bacterial growth rates (Fig. 2). As expected, with an increase in the doubling time of bacteria, the time of T4 latent period also increased and burst size decreased. In the case of the slowest analyzed growth rate of bacteria (μ = 0.033, which correlates with the doubling time of about 21 hours), the burst size was close to 1 phage per infection center. This suggests that the μ = 0.033, or the doubling time = 21 h, was close to the borderline growth rate of *E. coli* for the development of phage T4. This contrasts with the mathematical model presented previously (Rabinovitch *et al.* 2002), where such a value was estimated to be about 160 min.

Discussion

Strategies employed by bacteriophages to adjust their development to bacterial hosts are fundamental to the persistence of phages in their natural environment (Brüssow, 2013). Depending on the physiological state of bacterial host

cells, phages can initiate various developmental mechanisms, all of which influence the dynamics of bacterial populations in an ecological niche (Fuhrman, 1999; Wommack & Colwell, 2000; Abedon *et al*., 2001; Suttle, 2007; Clokie *et al*., 2011; Golec *et al*., 2011). In a standard laboratory culture, bacteria have all the factors essential for an optimal growth. These conditions could be called "a five-star hotel with all inclusive options". They are, however, rarely found in the natural environment. Thus, understanding of phage-host interactions in natural habitats should be, from our point of view, one of the most important research directions in phage biology.

Adsorption to the surface of a bacterial cell is the first step in a phage life cycle. Efficiency of adsorption depends on many physical factors in the environment as well as on the number and size of the host cells (Storms *et al*., 2010). For the adsorption process, phage T4 requires monovalent cations at the minimum concentration of 10 mM and the temperature in the range of 37 – 40°C (Kutter *et al*., 1994). Although conditions in a mammalian intestine - the natural environment for T4 - fulfill these requirements, it is important to stress that bacteria normally grow there significantly slower than under laboratory conditions (Kutter *et al*., 1994). Slow growth of bacteria influences the size of bacterial cells, making them smaller than in the case of fast growing laboratory cultures (Pierucci, 1978). Hadas *et. al*. (1997) showed that with a decrease in growth rate of bacteria, adsorption of T4 is less efficient. It should be noted, however, that Hadas *et. al*. (1997) used various kinds of media to manipulate the kinetics of bacterial growth.

In contrast to the experimental approach employed in the study by Hadas *et. al*. (1997), our experiments on kinetics of T4 adsorption to slowly growing bacteria were carried out with the use of a chemostat system that enabled us to control the

bacterial growth rate precisely, with no changes in the medium composition. We observed no significant differences in the efficiency of T4 adsorption on *E. coli* cells growing at μ ranging from 0.033 to 0.3, which we used in this work. Moreover, 10 minutes after infection by T4, the adsorption efficiency was at the same level for all the growth rates used in the experiments. We explain this phenomenon by the fact that the bacterial surface area and the number of receptors on its surface were similar in bacteria that grew with different μ in the chemostat.

After adsorption of T4 and injection of its DNA into the host cell, phage T4 takes control of the bacterial metabolic machinery and starts to produce progeny phage particles (Miller *et al*., 2003). T4 is able to control its development in response to bacterial physiology (Hadas *et al*., 1997). It is able to prolong its life cycle when it infects bacteria with a prolonged growth rate. To date, there are only a few reports on development of T4 in slowly growing host cells. Theoretical model of this phenomenon, which was based on only six different growth rates, suggested that T4 is unable to develop in bacteria whose doubling time was longer than 160 minutes (Rabinovitch *et al*., 2002). However in that study, the use of different media might have had a major effect on differences in metabolism of bacterial cells as well as on the phage development (e.g. influencing the process of adsorption). In this work we demonstrated that T4 is able to effectively complete its life cycle in bacteria with doubling times significantly longer than those predicted with the use of the theoretical model (Fig. 2). Duration of one T4 developmental cycle, determined in a modified one-step growth experiment, was longer than under standard laboratory conditions and longer than described by Rabinovitch *et al*. (2002). We still observed development of T4 in bacteria with $\mu = 0.033$ (which correlates with a doubling time

of about 21 hours). The burst size was then about 1 phage progeny particle per 1 infected bacterial cell (Fig. 2), suggesting that this μ is close to the actual limit for T4 development in a medium with glucose as a sole carbon source.

The ability to adapt phage's development to various physiological states of *E. coli* is not limited to phage T4. You *et al*. (2002) analyzed production of phage T7 daughter particles in *E. coli* cells at their different physiological states using a continuous culture and various dilution rates. They found a similar phenomenon that is observable in the case of T4. When growth rate of the host decreased, the eclipse time increased and burst size was reduced. Data presented by You *et al*. (2002) and our work suggests that developmental adaptation of lytic bacteriophages to host cell status is much more common than previously anticipated. Control of development of T4 in bacteria growing under conditions which are similar to those of natural environment is still poorly described. On the other hand, one can suggest that the knowledge about development of phages in slowly growing bacteria is crucial for understanding phage-driven ecosystems. In the natural environment, bacteria very rarely meet conditions that do not limit their growth. Therefore, phages which coexist with bacteria in every kind of environment must use various developmental mechanisms to carry out effective development. In our opinion, in the light of surge of phage application as therapeutic factors and of growing evidence of their destructive role in the biofermentation industry, it is crucial to focus on understanding phage-bacteria interactions under conditions occurring in their natural habitats.

Acknowledgements

The authors would like to thank Katarzyna Potrykus for critical reading of the manuscript. This work was supported by the “Iuventus Plus” Grant IP2011 015071 from the Ministry of Science and Higher Education (Poland) to P.G.

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Fig. 1. Efficiency of adsorption of bacteriophage T4 on *E. coli* cells growing at different rates. Bacterial cultures were infected with T4 at m.o.i. = 0.1, and at indicated times after infection the number of unadsorbed (remaining in the supernatant following centrifugation) phages was determined by plating, providing a basis for calculation of % of adsorbed phages. Particular columns correspond to growth rates shown on the picture. The results presented are mean values from 3 independent experiments with error bars representing SD. Statistical analysis (t-test) indicated no significant differences between any values compared at the same time after infection ($p>0.05$ in all tested combinations for all tested times; the values between different time points were not compared).

Fig. 2. Dependence of the burst size (open triangles; left Y axis) and latent period (closed squares; right Y axis) of phage T4 on *E. coli* growth rate. Bacterial cultures grown in a minimal medium at 37°C in a chemostat were infected with phage T4 at m.o.i. = 5 (at time = 0), and burst size and latent period were estimated at indicated times on the basis of results of phage titration from chloroform-treated culture samples. The results presented are mean values from 3 independent experiments with error bars representing SD.