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

Piotr Golec¹, Joanna Karczewska-Golec², Marcin Ło⁰²·³ & Grzegorz Wegrzyn²

¹Laboratory of Molecular Biology (affiliated with the University of Gdańsk), Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Gdańsk, Poland; ²Department of Molecular Biology, University of Gdańsk, Gdańsk, Poland; and ³Institute of Physical Chemistry, Polish Academy of Sciences, Warsaw, Poland

**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 the 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 10³¹ particles and is 10 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 c. 10²⁵ 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 (Clark & March, 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; Suttle, 2007). 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, for example, lytic or lysogenic cycle, pseudo-
sogeny or lysis inhibition (LIN). 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 min (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 & Wegrzyn, 2012; Cenens et al., 2013), LIN (Dressman & Drake, 1999; Golec et al., 2010) or prolongation of its development when bacterial growth rate (referred to as μ) increases (Rabinovitch et al., 2002; Golec et al., 2013).

The duration of phage T4 latent period depends on the bacterial μ (Hadas et al., 1997; Rabinovitch et al., 1999, 2002; Abedon et al., 2001; 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 min and led Rabinovitch et al. (2002) to ask whether any bacterium can release phages when its doubling time is longer than 160 min. Results of our recent experiments on the development of T4 in slowly growing *E. coli* indicated that T4 produces progeny particles in bacterial cultures with doubling times significantly longer than 160 min (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 the 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**

*Escherichia 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 the 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 as follows: 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)] was 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 milliliters 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 MgSO₄) were prepared. Two and half microliter of serial dilutions was spotted onto a bacterial lawn. Plates were incubated at 37 °C, and plaques were counted after 16 h.

**Efficiency of phage adsorption**

One milliliter 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 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 milliliters of the bacterial culture from the stabilized chemostat culture was 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 g for 1 min at RT, resuspension in FB medium prewarmed 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’ concentrations, pH value, and temperature (Storms et al., 2010). In this study, we analyzed kinetics of adsorption of phage T4 to a 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 the development of phage T4 in slowly growing bacteria

Chemostat cultures of E. coli were used to examine the differences in kinetics of the 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 h), 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, without 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 min 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 the development of T4 in slowly growing host cells. Theoretical model of this phenomenon, which was based on six different growth rates, suggested that T4 is unable to develop in bacteria whose doubling time was longer than 160 min (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 the development of T4 in bacteria with μ = 0.033 (which correlates with a doubling time of about 21 h). The burst size was then about one phage progeny particle per 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 the 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 the 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 the 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.

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