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The initial adsorption of T4 bacteriophages to *Escherichia coli* cells at equivalent concentrations: Experiments and mathematical modeling

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ABSTRACT

The emergence of antibiotic-resistant mutants among pathogenic bacteria has re-focused interest in alternative antibacterial treatments such as "phage therapy", where viruses are harnessed to infect and destroy bacteria included in their host range. The first stage in bacteriophage multiplication, its adsorption to the bacterial cell surface, has not been accurately resolved before. Previous studies focused on very low phage-to-bacteria concentration ratios. In this study, detailed kinetics of T4 adsorption to *Escherichia coli* B/r were obtained with high sampling frequency during the first 6 min, with suspensions of nearly the same initial number of phages and bacteria. The results were used to analyze several optional models and to choose the most suitable, based on simplicity and best fit to the data. It was found that simple, mono-attachment models adequately fit the experimental data.

1. Introduction

Since the middle of the 20th century, many of the infectious diseases caused by bacteria have been treated effectively with antibiotics, and it was hoped that these diseases would cease to pose threat to mankind [1]. However, mutants resistant to commonly used antibiotics emerged in recent years among bacterial pathogens that had been extensively treated with antibiotics. The promising potential of phage therapy [2] as an alternative to antibiotics has stimulated research on viral multiplication in bacteria in general, and during its early adsorption phase [e.g., 3, 4] in particular.

Consequent to simplicity and convenience in experimental protocols, the species *Escherichia coli* became the "model cell" in bacteriological research [4,5]. In the 1940s and 1950s, phages of the T series were commonly used as model systems for viral infection. Mathematical models describing the complete multiplication cycle appeared in the 1990s [6,7], but certain questions about the adsorption stage remained unsolved. Recent publications [4,8] also seem to overlook the complexity of this stage.

The cell envelope of Gram-negative bacteria such as *E. coli* [9,10] consists of peptidoglycan polymer wrapped by an outer membrane of lipopolysaccharides (LPS) and a cytoplasmic membrane at the inner side. Both membranes have various types of embedded proteins. There is little doubt about the role of LPS in T-phage attachment [11], to which phages attach by "tail fibers", but there are probably several other factors and components on the cell envelope that serve as phage-receptors [12,13]. Following attachment to LPS, the phage anchors irreversibly [14] and subsequently injects its DNA into the bacterium [15]. Once the DNA is inside, intracellular mechanisms are employed to synthesize phage components. The initial phages-to-bacteria ratio is referred to as multiplicity of infection, *MOI* [3,7,16]. Two *MOI*-based strategies are commonly used for culture infection by phages: High *MOI*, where the phages-to-bacteria ratio introduced to the bioreactor is high, and Low *MOI*, where this ratio is low.

Most previous studies applied Low *MOI* to ensure that no bacterium adsorbed more than a single phage [17]. The experiments described in this study were performed with *MOI* of approximately 1.

2. Theory

Attachment is the binding of phage to a bacterium that occurs upon their encounter. It can be reversible or irreversible. According to one model [18–20] reversible and irreversible attachments occur consecutively. According to another model [21,22], these two attachment types are mutually independent (see Section 5). *Injec-*

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tion of phage DNA into a bacterium is an irreversible event. Once the phage DNA is injected, the bacterium is considered *infected*. For convenience, irreversible attachment and injection are lumped into a single step, *adsorption*, thus bearing the implicit assumption that irreversible attachment is necessarily followed by injection. The *infection* process commences upon DNA injection and terminates when the bacterium lyses.

Puck and Garen [18,19] found that adsorption of several T-type phages to *E. coli* is ion-dependent. This finding was later supported by observations that phage tail fibers adhere to negatively charged regions in the LPS [11]. Additionally, adsorption of the T1 phage was found [19] to be temperature-dependent. Since electrostatic forces are not affected by temperature, it was conceived that other factors which *are* temperature-dependent also govern adsorption. Accordingly it was proposed that the adsorption process consists of two consecutive steps (also called 'two-step adsorption', or 'sequential adsorption' theory). The first is an ionic-dependent reversible attachment step and the second is a temperature-dependent irreversible attachment step (see next paragraph). It is important to note however that different phages have different adsorption mechanisms and may therefore operate differently [23].

2.1. Common kinetic models of adsorption

The most commonly known kinetic models of phage-to-bacterium adsorption are summarized below. The phage-free bacteria and the free (non-attached) phages are denoted by B and P, respectively. They react with each other in either a reversible or an irreversible manner. The resulting reversible and irreversible complexes are denoted by R and I, respectively. In the following schemes rate constants above and below the reaction arrows denote rightward and leftward reactions, respectively.

2.1.1. The Sequential model

The Sequential model [18–20] is described by the following scheme:

$$P + B \underset{k_2}{\overset{k_1}{\longleftrightarrow}} R \xrightarrow{k_3} I$$

Here, a reversible attachment is an essential precursor to an irreversible one. These works [18–20] demonstrated the reversible nature of the first reaction and that only irreversible attachments lead to bacterial death.

2.1.2. The Modified Sequential model

This model was proposed by Christensen [24] to explain experimental results that were inconsistent with neither the Sequential model nor the Competitive model (see below). It can be described by the following scheme:

$$R_b \underset{k_4}{\overset{k_2}{\longleftrightarrow}} P + B \underset{k_2}{\overset{k_1}{\longleftrightarrow}} R_a \xrightarrow{k_3} I$$

Here, R_a denotes a reversible complex that may potentially become irreversible I, and R_b denotes a reversible complex that does not yield an irreversible one.

2.1.3. The Competitive model

The Competitive model [21,22] is described by the following scheme:

$$R \underset{k_2}{\overset{k_1}{\longleftrightarrow}} P + B \xrightarrow{k_3} I$$

Accordingly, bacterial-phage encounters yield either adsorptions or reversible attachments.

The Modified Sequential as well as the Sequential and Competitive models assume mono-attachment scenarios, namely that every bacterium adsorbs a single phage at most—a reasonable assumption when the initial bacterial concentration B_0 is much higher than that of the phages ($MOI < 0.1$), as is the case in the investigations mentioned above.

The abundant experimental and theoretical studies on phage adsorption kinetics were devoted to low *MOI*s and performed with low sampling resolutions. The present research concerns infections of phages and bacteria at equivalent initial concentrations, monitored at high frequency in order to detect abrupt changes in the adsorption process.

Kinetic models were formulated to interpret the experimental results, aimed at obtaining the simplest formalism that could adequately describe the phage and bacterial concentrations during the early stage of the infection process.

3. Materials and methods

3.1. Bacterial growth and initiating experiments

The models described below were compared with experimental results obtained with wild type T4 phages infecting *E. coli* B/r (H266) cells [25]. The bacteria were cultured by shaking at 37 °C in phosphate-buffered salts medium M9 [26] supplemented with magnesium sulphate (1 mM), calcium chloride (0.1 mM) and glucose (0.4%). In a typical experiment, suspensions of phages and exponentially growing cells, each at an approximate concentration of 2×10^8 ml^{-1} were mixed together. Duplicate samples were drawn from the mixture vessel at intervals of 15–30 s for bacterial and phages counting, respectively. The samples were immediately diluted to prevent new phage attachments.

3.2. Counting bacterial cells

The samples allocated for bacterial counts were diluted in test tubes immersed in an ice water tub to prevent further multiplication of bacteria. To determine cell concentrations, the diluted samples were spread over LB-agar plates [26] and colonies were counted after overnight incubation at 37 °C. The colony-forming unit (*CFU*) counts and dilution details were used to retrieve concentration time series. If irreversible attachments follow reversible ones these time series reflect the concentration of free bacteria at the sampling instants. However, if reversible and irreversible attachments are competitive and mutually exclusive these time series reflect the inclusive concentration of free and reversibly attached bacteria.

3.3. Counting phage particles

To determine phage concentrations, the samples allocated for phage counts were diluted in tubes containing chloroform to burst the cells and release reversibly attached phages. The diluted samples were mixed with concentrated suspension of the *E. coli* B/r (H266) cells (indicator) and spread on LB-agar plates using soft agar [26]. After overnight incubation plaques (bacteria-free clearings) appeared on the indicator lawn, each originating from a single plaque forming unit (*PFU*). Consequently, the *PFU* counts and dilution details were used to retrieve the all-inclusive concentration time series of free and reversibly attached phages in the suspension.

4. Results

4.1. Proposed kinetic models

Mono-attachment models are presented and analyzed here. They are based on similar principles as those mentioned in Sec-

tion 1. Since the adsorption experiments were performed during the exponential growth phase [27], bacterial multiplication is also accounted for, approximated as a first order kinetic model:

$$\frac{dB}{dt} = \mu B, \tag{1}$$

where μ is the growth-rate constant. Given an initial bacterial concentration B_0:

$$B(t) = B_0 e^{\mu t} \tag{2}$$

4.1.1. Improved Sequential model

This model is similar to the Sequential model mentioned in Section 1 except for the additional bacterial growth term:

$$\begin{cases} \frac{dP}{dt} = -k_1 PB + k_2 R \\ \frac{dR}{dt} = k_1 PB - (k_2 + k_3)R \\ \frac{dB}{dt} = -k_1 PB + k_2 R + \mu B \end{cases} \tag{3}$$

As discussed in Section 3, *PFU*s originate from free and reversibly attached phages, and in this model *CFU*s originate from free bacteria. Hereinafter the abbreviations *PFU* and *CFU* will also represent the concentrations of these entities in accordance with the context in which they are mentioned. Here

$$\begin{cases} PFU = P + R \\ CFU = B \end{cases} \tag{4}$$

The growth-rate constant, μ was evaluated from growth curves in the exponential phase fitted to Eq. (2) (data not shown).

4.1.2. Improved Modified Sequential model

The model is based on the Modified Sequential scheme with the inclusion of the bacterial growth term:

$$\begin{cases} \frac{dP}{dt} = -(k_1 + k_4)PB + k_2(R_a + R_b) \\ \frac{dB}{dt} = \mu B - (k_1 + k_4)BP + k_2(R_a + R_b) \\ \frac{dR_b}{dt} = k_4 PB - k_2 R_b \\ \frac{dR_a}{dt} = k_1 PB - (k_2 + k_3)R_a \end{cases} \tag{5}$$

The relation between the measured *PFU* and *CFU* and the model variables may be summarized as:

$$\begin{cases} PFU = P + R_a + R_b \\ CFU = B + R_b \end{cases} \tag{6}$$

4.1.3. Improved Competitive model

The model is based on the Competitive model described in Section 1 with the inclusion of the bacterial growth term:

$$\begin{cases} \frac{dP}{dt} = -(k_1 + k_3)PB + k_2 R \\ \frac{dR}{dt} = k_1 PB - k_2 R \\ \frac{dB}{dt} = \mu B - (k_1 + k_3)PB + k_2 R \end{cases} \tag{7}$$

Here colonies represent the sum of free and reversibly attached bacteria:

$$\begin{cases} PFU = P + R \\ CFU = B + R \end{cases} \tag{8}$$

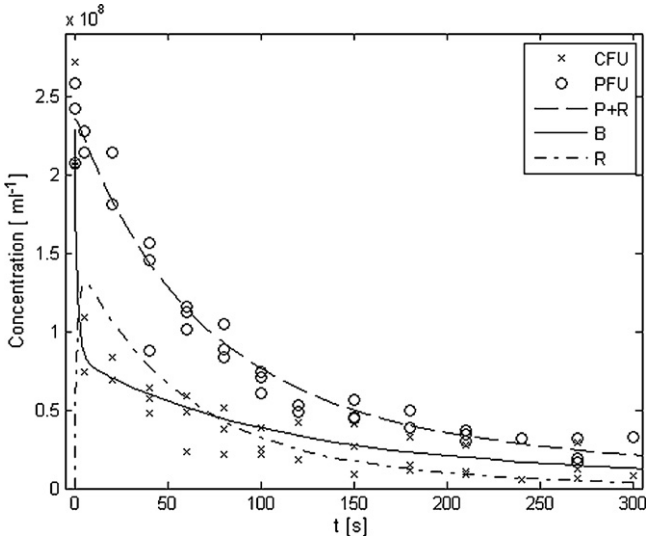

Fig. 1. Improved Sequential model. Measured concentrations of *CFU* (×) and *PFU* (○); three repetitions of the experiment yielded 1–3 readings at each sampling instant; curves indicate model predictions; *P* + *R* denotes free and reversibly attached phages; *R* denotes reversible complexes; *B* denotes free bacteria.

The ordinary differential equations describing each model were solved numerically by a 4th order Runge–Kutta method [28]. The parameters were calculated by fitting each model to the experimental data using the least squares approximation method. Programming was performed with the MATLAB© software.

The models were rated according to the sum of squared differences between model predictions and measured *PFU* and *CFU*.

4.2. Adsorption experiments and model predictions

Results of adsorption experiments and predictions of models 4.1.1–4.1.3 are shown in Figs. 1–3. The most striking feature observed is the initial sharp drop in *PFU* and *CFU*, followed by a moderate decline. *CFU* dropped much faster: from an initial value of 2.06–2.72 × 10^8 ml^{-1} to approximately 1 × 10^8 ml^{-1} in 10 s, whereas equivalent drop in *PFU* lasted over a minute. It took

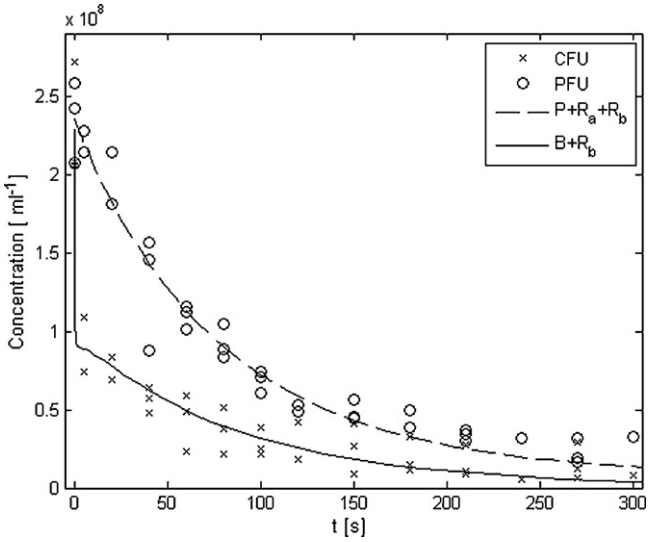

Fig. 2. Improved Modified Sequential model. Measured concentrations of *CFU* (×) and *PFU* (○); three repetitions of the experiment yielded 1–3 readings at each sampling instant; curves indicate model predictions; *P* denotes free phages; R_a denotes reversible attachments that can potentially become irreversible; R_b denotes reversible attachments that cannot become irreversible; *B* denotes free bacteria.

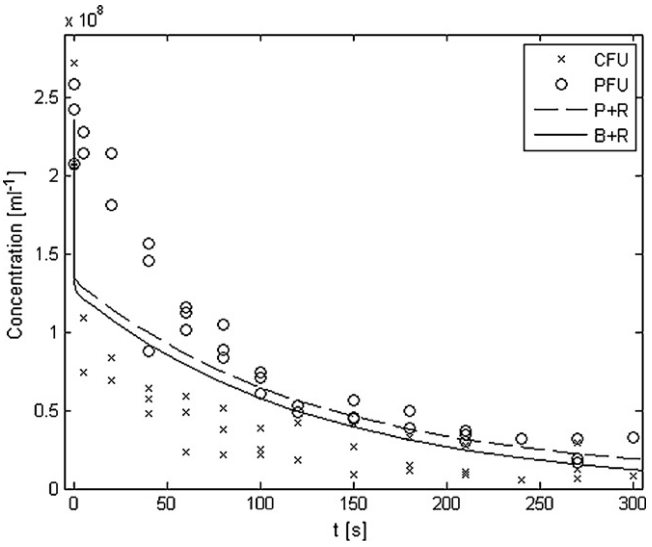

Fig. 3. Improved Competitive model. Measured concentrations of *CFU* (×) and *PFU* (○); three repetitions of the experiment yielded 1–3 readings at each sampling instant; curves indicate model predictions; *P* and *R* denote free phages and reversible complexes, respectively; *B* denotes free bacteria.

Table 1
Summary of models and their fit results.

Model	Equations	Sum of squares [ml^{-2}]
Improved Sequential	(3) and (4)	1.47×10^{16}
Improved Competitive	(5) and (6)	6.76×10^{16}
Improved Modified Sequential	(7) and (8)	1.54×10^{16}

the bacteria 1 min and the phages 2 min to reach concentrations of 0.5×10^8 ml^{-1}. In several experiments, in which the sampling period was prolonged to about 10 min (data not shown), *PFU* and *CFU* seem to converge to the same asymptote after the 5th minute.

Of the 3 proposed models, the Improved Sequential (Fig. 1) and Improved Modified Sequential (Fig. 2) fit well to the experimental data of both *PFU* and *CFU*. The sums of squares of these two models are approximately four times lower than the sum of squares of the Improved Competitive model (Fig. 3), as seen in Table 1. The fitted kinetic parameters of the Improved Sequential and Improved Modified Sequential models are shown in Table 2.

5. Discussion

This investigation was aimed at finding the simplest model that can represent concentration time-profiles of bacteria and phages during the attachment period. Obviously, multiple phage adsorptions to a single bacterium do occur, as clearly demonstrated by the existence of genetic recombination between phages and by the "Lysis from without" and "Lysis Inhibition" phenomena [29], but their relative role in the kinetics of the infection process itself remains unclear. However the inclusion of additional multi-attachment variables: single cells having two, three, or more attached phages would increase the complexity of the models significantly. Since these variables were not measured in our experimental work (and in fact cannot be measured by common techniques) they would add degrees of freedom to the system. All of the above arguments stimulated the motivation to test the adequacy of models based on single phage-to-bacterium attachments in characterizing the early stage of infection.

Table 2
Parameters of Improved Sequential and Improved Modified Sequential models fit to the experimental results.

Parameter	Best fit	
	Improved Sequential model	Improved Modified Sequential model
μ [s^{-1}] (fixed)	2.5×10^{-4}	2.5×10^{-4}
k_1 [ml s^{-1}]	2.27×10^{-9}	2.27×10^{-8}
k_2 [s^{-1}]	1.1×10^{-1}	1.01×10^{-1}
k_3 [s^{-1}]	2.21×10^{-2}	2.29×10^{-2}
k_4 [ml s^{-1}]		1.13×10^{-8}

The main variables of interest in this study are the concentration profiles of free bacteria *B* and free phages *P*. Among the investigated models only the Improved Sequential and Improved Modified Sequential (Eqs. (3)–(6)) show good correspondence between the variable *B* and measured *CFU* (Figs. 1 and 2). Fig. 1 shows the best fit obtained for the Improved Sequential model with respect to the experimental data. The model describes the evolution of each of the composites during the early stages of the infection, as well as the virtual reversible complex, *R*. The reasonable predictive quality of the model with respect to the measurable variables corroborates the assessment of *R*.

Fig. 2 shows the best fit obtained for the Improved Modified Sequential model. Two intermediate model variables, R_a and R_b are presented in the figure.

The Improved Sequential model yields only a slightly better fit than the Improved Modified Sequential model, as reflected by the sum of squares in Table 2. This near equivalence is insufficient to decisively determine which of the two is better. We may apply Occam's Razor and favor model 4.1.1 (Improved Sequential model), based on its simplicity. The predicted profile of $P+R$ in this model fits quite well to its measured values (*PFU*).

Fig. 3 displays the best fit obtained for the Improved Competitive model. The inadequacy of the model is evident, as also confirmed by the statistics in Table 1: its sum of squares is over fourfold higher than that of the former two and is therefore rejected.

Christensen [24] proposed the Modified Sequential model because his experimental data were inconsistent with the Sequential and Competitive models. The difference between his predictions and the present results could also be attributed to the different adsorption properties of the T1 and T4 phages. For example, while T1 and ϕ80 require energy for irreversible adsorption, the other phages of the T series do not. T4 needs no external energy source for irreversible adsorption; it can eject its DNA upon adsorption to bacterial wall debris [30].

The high sampling rate at intervals of 15–30 s is a clear improvement in resolution over previous studies, where sampling intervals were of several minutes. In particular, it plays an imperative role in resolving the abrupt changes during the early stages of the infection. The *PFU* and *CFU* profiles show a very sharp drop, followed by a much more temperate descent after the first 10 s. It is also noticed that *CFU* drops faster than *PFU*. This complex behavior can be explained by performing an order-of-magnitude analysis on the Improved Sequential model. Initially the bacteria and phage concentrations are of the same order of magnitude ($\cong 2.3 \times 10^8$ ml^{-1}) and the concentration of the reversible complex, *R* is zero. With $k_1 = 2.27 \times 10^{-9}$ ml s^{-1}, $k_2 = 1.1 \times 10^{-1}$ s^{-1}, $k_3 = 2.21 \times 10^{-2}$ s^{-1}, $\mu = 2.5 \times 10^{-4}$ s^{-1} it follows from Eq. (3) that

$$\frac{d(P+R)}{dt} = -k_3 R \gg -k_1 B^2 + k_2 R + \mu B \cong -k_1 B^2 \cong -10^8 \cong -k_1 PB + k_2 R + \mu B = \frac{dB}{dt}$$

Consequently, immediately after mixing the bacteria and phages, the forward reaction rate is fastest, the reverse reaction is negligible, bacteria and phage concentrations drop and the concentration of the reversible complex *R* builds up quickly and reaches its maximum (1.4×10^8 ml^{-1}) after ~6 s. Subsequently, the combined effect

of *R* dissociation (to free phages and bacteria) and the reduction in phage–bacteria attachments yield the moderate slope in *CFU* and *PFU*.

The fact that mono-attachment models adequately fit the experimental results does not rule out the occurrence of multiple attachments. In fact, multiple attachments do occur at higher values of *MOI* [12,17]. Nonetheless the good fit observed here clearly suggests that near *MOI* = 1, the simple Improved Sequential model provides reasonable predictions of bacteria and/or phage concentrations upon their mixing.

Future studies will be done to explore the ranges of cells and phages for which the Improved Sequential model remains valid.

6. Conclusions

Based on sequential adsorption theory it is shown that a simple mono-attachment model can be used to suitably describe the kinetics of phage attachment to susceptible bacteria at *MOI* = 1. To the best of our knowledge, the Improved Sequential model introduced here is the simplest model that successfully describes adsorption experiments under such circumstances. Eventual implementation of phage therapy will require large scale production of phages. The present model becomes a useful design tool for processes in which phage attachment is the rate-limiting factor.

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