T4 Bacteriophage Average Burst Size Varies with *Escherichia coli* B23 Cell Culture Age

Madison Bolger-Munro, Kelvin Cheung, Anne Fang, and Lisa Wang
Department of Microbiology and Immunology, University of British Columbia

Host physiology has a dramatic effect on the ability of T4 bacteriophage to infect and produce progeny. Previous studies have shown that as *Escherichia coli* cells enter stationary phase, many physiological changes to cell size and shape occur. These changes could affect the ability of the T4 bacteriophage to produce progeny. In this study, standard plaque assays were used to investigate the effects of culture age on phage burst size. Cells were grown for 1, 3, 4 and 5 days in liquid H media and infected with T4 bacteriophage. Burst size from day 3 cultures was approximately half of the overnight culture’s burst size, burst sizes from day 4 cultures were lower than that of day 3 by approximately 10-fold and burst sizes from day 5 cultures were greater than day 4 by approximately 350-fold. No overall decreasing trend was seen in burst size perhaps due to biofilm production. The small burst sizes seen in the day 3 and 4 of stationary phase may be due to weakening phage adsorption from altered cell shape. The large burst size seen in day 5 of stationary phase may be due to altered adsorption efficiency from biofilm production or due to the culture undergoing two lytic cycles. The day 5 cultures showed larger plaques, which may be due to inconsistent indicator bacteria concentration or indicator bacteria not being in exponential phase of growth.

Understanding bacteriophages and their interaction with host cells gives insight into molecular biology and may lead to an improved understanding of treatment methods against bacterial infection. T4 bacteriophage adsorption occurs when the long tail fibres of the phage attach to the Omp proteins located on the surface of an *Escherichia coli* cell (1). Since the initial binding of the T4 bacteriophage to the *E. coli* cell is weak, both cell size and cell shape have been implicated in altering the stability of adsorption and thus the susceptibility of *E. coli* to bacteriophage infection (2). When in stationary phase, *E. coli* cells are starved of nutrients and thus the cell size decreases and the cells become more spherical rather than rod-like in shape (3, 4). When cells are infected by a lytic phage and enough phage parts are synthesized and assembled, the phage will trigger a molecular switch in the host cell to produce lysis, an enzyme that degrades the cell wall, causing the release of phage particles (5). This process is called lysis. The average number of phage particles released by a cell after one infection cycle is called the average burst size (6). Burst size varies substantially from a few particles to a few thousand (7). Investigations into viral burst sizes have been performed by using the T4 bacteriophage as a model. T4 bacteriophage is an appropriate model system for our study because it has been shown to produce consistent plating results and its molecular biology is understood very well. Also, T4 bacteriophage is easy to work with and has a rapid lytic cycle, thus establishing it as a good tool for studying T-even phages.

Currently, investigations into burst size variability have been inconclusive. Ellis and Delbrück (8) postulated that burst size increases as a function of infection time; bursts that resulted after a short incubation time would be smaller than those that resulted after a longer incubation time. However, examination of growth plates grown for the same length of time still exhibited a variation in burst size. As a result, Ellis and Delbrück (8) hypothesized that phage particles were not released simultaneously, and thus showed variation. However this was not proven. Another hypothesis to explain the variable burst size phenomenon was that bacterial cells infected with more than one phage particle would produce larger bursts but this was disproved as well (8). Choi et al. (9) studied the effect of cell size on burst size. Their hypothesis was that larger cells have more machinery to produce more phage particles, thus creating a larger burst size. The results from their study showed that cell size did have an effect on burst size. They also observed that the largest cells did not exhibit any phage bursts. They proposed that there was a critical cell size after which the correlation between cell size and burst size ceases. Variability of burst size is still not fully understood.

The aim of this study is to investigate the effect of culture age on the phage burst size. The aforementioned findings from previous studies stated that stationary phase cells in particular have lower adsorption efficiencies than exponential phase cells (9). As such, the amount of cells initially infected by phage should decrease with the age of the culture; hence the average burst size would be expected to decrease. This effect was observed with the stationary phase day 3 and 4 cultures as adsorption efficiency decreased by approximately 350-fold. However this difference in adsorption only resulted in a 10-fold difference in average burst size. The number of phage adsorbed for the day 5 stationary phase *E. coli* culture in comparison to the day 4 stationary phase culture increased by 15-fold, instead of the expected decrease in adsorption efficiency. This resulted in a 350-fold greater average burst size in day 5 stationary phase *E. coli* than in day 4 stationary phase cells.

**MATERIALS AND METHODS**

Preparation of bacterial strains and reagents. The LPS mutant *E. coli* B23 and ompA JW0940 mutant strains were obtained from the UBC Department of Microbiology and Immunology (Vancouver, Canada). The *E. coli* cultures were streaked and
grown overnight on 1.5% w/v H agar plates. A stock culture was made by inoculating a pure colony into 20 ml of liquid H media (1.0% w/v tryptone and 0.8% w/v NaCl). Investigation of the average burst size of T4 bacteriophage was carried out using E. coli B23 as the host cell for infection. The cells were cultured in liquid H media and grown at 37°C in a shaking water bath.

Preparation of viral stock and stock concentration determination. T4 bacteriophage was obtained from the MICB 421 Culture Collection in the Department of Microbiology and Immunology at the University of British Columbia (Vancouver, Canada). The method for viral stock preparation was adapted from Braun et al. (10). To prepare a new viral stock, an overnight culture of E. coli B23 cells was infected with T4 bacteriophage. After an overnight incubation in a 37°C shaker, cell debris was pelleted by centrifugation at 5000 RPM for five minutes. Free phage particles were recovered in the supernatant and 5 μl of chloroform was added to prevent microbial contamination (11). The viral titer was determined by plaque assays, which was adapted from the Molecular biology of bacteriophage T4 (12).

Preparation of stock. First, the phage stock was serially diluted to 10^7, 10^6, and 10^5 dilutions, respectively. Then, 0.25 ml, 0.1 ml, and 0.05 ml of the 10^7 dilution were each added to top agar mixtures and subsequently plated. These plates were counted and the viral concentration used for the stationary phase as well as the single cycle burst size growth assays, only plates with 30 to 300 plaque forming units (pfu) were taken into consideration when determining the burst size (14).

Pour plating. Soft-overlay plates were prepared for pour plating with 1.5% w/v H agar in the bottom layer and 3.5 ml of 0.7% w/v H agar in the top layer. Undiluted B23 at a volume of 0.2 ml was added to the top agar as indicator cells. The top agar was incubated at 48.5°C water bath, which slows the growth of the E. coli cells, but does not kill them (13). All top agar mixtures were mixed via phage style mixing, which involves circular rotation of the test tube with the direction of rotation perpendicular to the ground. The top agar mixture was poured onto 1.5% w/v H agar plates and incubated overnight at 37°C. Plaques were counted the following day. For the average burst size growth assays, only plates with 30 to 300 plaque forming units (pfu) were taken into consideration when determining the burst size (14).

Control plating. E. coli JW0940 is characterized by a deletion of ompA, which encodes a receptor for many T-even E. coli phages. Due to this deletion, JW0940 would not be infected by bacteriophage and thus bursts would not occur. To test for bacteriophage resistance in the JW0940 strain, plaque assays were made from JW0940 and B23 cultures and compared to each other. No plaques formed on the JW0940 plates because it is resistant to T4. The B23 plates contained plaques because this strain is susceptible to T4 infection.

Bacteriophage growth curve assays in E. coli B23 in day 3, 4, and 5 stationary phase and log phase. Burst size assay procedures were adapted from the MICB 323 Laboratory Manual (6). This procedure was modified to use JW0940 cells as a diluent for the stationary phase B23 cells to prevent B23 reabsorption into log phase. Liquid H media was used as a diluent for the log phase B23 cells. One ml of B23 culture was inoculated into 20 ml of liquid H media to produce a starter culture. The B23 cells for the exponential phase assay were grown overnight for approximately 24 hours prior to the experiment, while the stationary phase B23 cells were grown for 3, 4, and 5 days. At the start of the experiment, the turbidity of the cultures were determined at a wavelength of 660 nm. The cultures were diluted to 10^6 cells/ml in order to achieve a multiplicities of infection (MOI) of 0.1, which ensures that each B23 cell was infected by one phage particle (6).

Determination of average burst size. The average burst size was calculated using two different equations. The first equation, which was adapted from the MICB 323 Laboratory Manual (6), takes into consideration the maximum phage yield (T50) and the amount of unadsorbed and adsorbed phage:

\[
\text{burst size (geometric mean)} = \frac{\text{maximum phage yield (T60 or T70)} \times \text{initial phage yield (T0)}}{\text{infected cells (T0 cell pellet)}}
\]

The second equation takes into consideration the maximum phage yield and initial amount of phage at T0:

\[
\text{burst size (geometric mean)} = \frac{\text{maximum phage yield (T60 or T70)} \times \text{pfu}}{\text{initial phage yield (T0) \times \text{infected cell}}}
\]

To compare the average burst sizes obtained from the two equations, the percentage difference was calculated:

\[
\text{percentage difference} = \frac{\text{percentage of equation 2 value} - \text{percentage of equation 1 value}}{\text{equation 1 value}} \times 100\%
\]

RESULTS

Sporadic sampling did not agree with expected trend for log phase growth curve. Figure 1 showed the bacteriophage growth curve for E. coli B23 cells in exponential phase. There appeared to be sporadic sampling points with no obvious trend. This observation did not agree with the trend that was expected, which was depicted in Fig. 1. As proven with numerous other studies, growth curves with exponential phase cells have distinct eclipse periods and log periods. In this experiment, the viral titer at T3 and T15 were approximately 0.07 × 10^6 pfu/ml and 0.04 × 10^6 pfu/ml more concentrated than expected. As a result, an eclipse period was not observed. If the viral titer at T5 was approximately 0.04 × 10^6 pfu/ml and T15 was 0.05 × 10^6 pfu/ml, then an eclipse period would have been observed. Moreover, a log period was not present (Fig. 1). The viral titer at T30 was approximately 50% of the expected value (0.3 × 10^6 pfu/ml); otherwise the log period would have been present. The expected burst values were determined based on where each sampling point would have fell on the curve (Fig. 1). A T30 sampling point was never measured for the exponential phase.
phase assay because the burst size appeared to level off at about 50-minutes post-infection.

**Stationary phase growth curves varied.** Even though sampling at 70-minutes was done for the day 3, 4, and 5 stationary phase growth curves (Fig. 2), only the day 3 curve displays $T_{r0}$ because $T_{r0}$ of the day 4 and day 5 experiments did not meet the 30 to 300 plaque count requirement outlined in the Materials and Methods section. The day 5 stationary phase curve showed a steeper rise period that extended across 40 minutes, while the day 3 and 4 stationary phase curves were observed in shorter time intervals of approximately 10 minutes. The rise period for the day 3 and 4 stationary curves were also not as steep as the day 5 stationary curve. In the alternative interpretation of the day 5 stationary phase growth curve (Fig. 3), a plateau was observed from 30-40 minutes with a rise period following that plateau. The viral titer was observed to begin to plateau again at 60-minutes. This differed from the expected one observed plateau and rise period for a one-step growth curve.

**Average burst size varied between log phase and stationary phase E. coli B23.** As shown in Table 1, the two equations used to calculate the average burst size gave differing results. With the exception of the day 5 stationary phase assay, there was a decreasing trend in the average burst size in correlation to the increasing starter culture age. For the exponential phase and day 3 stationary phase average burst sizes, both equations gave similar results, as indicated by the small value of the percentage differences between the results of the two equations. For the day 4 stationary phase average burst sizes, both equations gave largely differing results. Interestingly, the result from equation 1 of day 4 stationary phase was a negative value. Based on the formulation of equation 1, a negative average burst size can only arise when the amount of unadsorbed phage is greater than the maximum yield. The average burst size calculated from both equations for the day 5 stationary phase assay was more than 10-fold larger than the other three assays. The $T_0$ and adsorbed phage burst values both decreased with the age of the culture, with exceptions in the day 5 culture. The $T_0$ and $T_{r0}$ adsorbed burst sizes decreased by more than 2-fold when the culture aged from day 1 (log phase) to 3 days. A further decrease of more than 150-fold for $T_0$ and 350-fold for $T_{r0}$ adsorbed occurred as the culture aged from day 3 to 4. As the culture aged from 4 to 5 day, the $T_0$ increased by approximately 10-fold and the $T_{r0}$ adsorbed increased by approximately 15-fold.

**DISCUSSION**

The differences in average burst sizes between stationary day 3, 4 and 5 cells were intended to show how culture age affected burst sizes. There was no evident decreasing trend in burst size seen over the three days. The day 5 burst size (FIG. 2.) was significantly greater than day 3 and 4, which agreed with the findings of two previous studies, which showed an increase in phage burst size in day 4 and 5 (15, 16). Chan et al. suggested that the increase in burst size at this time corresponded to the formation of biofilm (16). Our cultures were aerated and constantly agitated at 200 rpm, which was presumed to prevent biofilm formation, but Hancock et al. suggested that biofilms can still grow well in shaking glass tubes (17). Our findings were consistent with this observation that biofilm formation was correlated with an increased burst size, but an explanation for how biofilm formation affects burst size has not been discovered yet.

Two equations were used to measure average burst size because significant variation was seen on the $T_{r0}$ and $T_0$ time point plates in days 3, 4 and 5. Equation 1 took into account the $T_{r0}$ plate plaque counts to measure average burst size. The number of plaques may have varied due to different adsorption efficiencies on different days, so the values inputted into equation 1 may have skewed the average burst size. Therefore, the original inputted viral concentration was considered to compare the results with those of the average burst size plate count calculation in equation 1.
The plaques observed on the day 5 stationary phase plates were much larger than the previous two days. There may be numerous reasons for this phenomenon. Firstly, cell density and turbidity measurements of the indicator bacterial culture was never measured prior to the start of the plaque assay experiments. Therefore, the concentration of indicator bacterial cells was not consistent in any of the plaque assays. The virus in day 5 may have been plated with a lower density of indicator bacteria than the other 3 assays. This would have resulted in earlier phage adsorption and consequently earlier bursts, thus larger plaques and a greater variation in timing of the adsorption of each phage resulted. High indicator bacteria concentrations may have led to a faster rate of division and establishment of stationary phase cells in the lawn, thus smaller burst sizes and plaque sizes was yielded (18).

Secondly, the growth phase of the indicator culture can affect the plaque size. The indicator culture should have been in exponential phase prior to the start of the experiment to yield ideal results (18). Cells in non-exponential phase cultures would change its physiology to re-enter exponential phase once they are inoculated into the top agar. During this transition period, plaque formation would be affected. The indicator bacterial cultures used in our experiments were all in non-exponential phase, thus variation in plaque sizes was observed. The third reason was the concentration of the top agar, which can affect plaque size (18). However, all top agar used in the plaque assays were 0.7% w/v in concentration, thus it was unlikely that this was the reason for the observed larger plaque size in the day 5 assay.

Aside from the day 5 stationary phase culture, the average burst sizes of the stationary phase cultures were less than that of the exponential phase culture. We suspected that cells in stationary phase have altered cell shape and thus weakening phage adsorption to the cells. This led to less infection of cells, which ultimately resulted in a smaller burst size. When the amount of adsorbed phage at $T_0$ was compared between the four assays, it was evident that the stationary phase cultures, with the exception of day 5, had less adsorbed phages than the exponential phase culture. The same concentration of starter culture and phage was used for all four assays to ensure that these parameters did not affect observed amount of adsorbed phage. The only exception to this trend was in day 5 stationary phase. It was suspected that the previously mentioned effect of biofilm formation in the day 5 culture altered the adsorption efficiency and thus the observed amount of adsorbed phage.

Another possible explanation for the extremely high burst size in the day 5 culture is that during the 70-minute sampling time, the infected culture underwent two lytic cycles. In Figure 3, there appeared to be a plateau followed by exponential growth that leveled off between 30-40 minutes. After this plateau, another exponential curve began. Hadas et al. have shown that lysis time, or the amount of time required to complete one lytic cycle, is affected by cell dimensions (19). T4 bacteriophage lysis time is negatively correlated with growth rate (20). Since it is known that cell growth rate in stationary phase is much lower than that of exponentially growing cells, the lysis time in the day 5 cultures was expected to be shorter. Furthermore, since day 5 might have marked the beginning of biofilm presence in the cultures, and growth rate in biofilms is reduced compared to planktonic cells, we expected lysis time to be much shorter than that of even the other stationary phase cultures. For this reason, it was possible that the lysis time for day 5 cultures was closer to 40 minutes and over the 70-minute sampling time two lytic cycles were observed. If this was the case, then in the first lytic cycle, the burst size would have been expected to be 32 pfu/cell (from equation 1) or 23 pfu/cell (from equation 2). While this was still a higher burst size than the other stationary phase cultures, it was consistent with other studies finding that day 4-5 stationary phase cultures have an increased burst size (15).

In conclusion, although a decreasing trend was observed in burst size from the exponential phase, stationary phase day 3, and stationary phase day 4 cultures, an overall decreasing trend which includes the stationary phase day 5 culture was not observed. The same result was also observed for T4 adsorption efficiency. This may have been due to biofilm production and more research will need to be conducted to determine the cause of the lack of an overall decrease in average burst size and adsorption efficiency.

**FUTURE DIRECTIONS**

Previous experiments showed that larger cells have larger burst sizes (9). Investigating the single cell burst sizes of larger exponential phase cells versus smaller stationary phase cells was a method to determine how the variation in burst size arose. This was the original purpose of the experiment. However, the single cell burst size experiments were inconclusive as the number of plaques seen on the plates did not coincide with expected values calculated from the total burst size experiments. Although the burst size determined by the growth curve of bacteriophage in exponential phase cells was found to be higher than the growth curve of bacteriophage in day 3 stationary phase cells, the single cell burst size experiment plates revealed very low number of plaque containing plates: the exponential phase assay produced 2 plaque containing plates out of a total of 52 plates, while the 3 day
stationary phase assay produced 1 plaque containing plate out of 52, and the 4 and 5 day assays produced 0. In these single cell burst size assay experiments, the phage infected B23 cells were incubated at room temperature without agitation. Instead, it would be imperative for the cells and phage to be incubated in a shaking water bath at 37°C because phage replication and the number of infected cells decreases drastically at temperatures lower than 37°C and without agitation, although the cells would still grow (21). The reasons for the low burst sizes may be because the cells were incompletely burst, or the observed viral plaques that were on the plates was a free phage, or the cells died prematurely. For more accurate and consistent results for the plaque counts and plaque sizes, the same concentration of E. coli B23 indicator culture should be used in all of the assays. Moreover, starter cultures for the exponential phase growth curve should be in mid-log phase (0.3 A600nm) prior to the start of the experiment (22).

If the single cell burst size assays had been successful, it would have been expected that the stationary phase assays would have produced less plaques than the exponential phase assay. The proposed explanation for this phenomenon was that there was less phage progeny produced in stationary phase cells because of the lower amount of cell machinery and consequently slower protein synthesis in stationary phase (23). Results from a single cell burst size experiment for log phase and stationary phase E. coli cells could possibly determine whether the variation in average burst size was due to either a decrease in the number of phage progeny released by each of the infected cells or a variation in the amount of progeny released among individual cells.

ACKNOWLEDGEMENTS
This study was supported by the Department of Microbiology and Immunology at the University of British Columbia, Vancouver, Canada. We would like to thank Dr. William Ramey for his help and guidance and Patrick Taylor for his constant support. Finally, we would like to express our gratitude to Lando Robillo, Nick Cheung, and others at the Wesbrook media room for providing all of the necessary laboratory supplies.

REFERENCES
14. Benbasat J. 2011. Microbiology 322 microbiological techniques I. Department of Microbiology and Immunology, University of British Columbia.