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Culture Age and Extrinsic Factors Affect Formation of T4 Bacteriophage Infective Centers in Stationary Phase *Escherichia coli* ZK126

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Studies of *Escherichia coli* performed using fresh or log phase cultures may not accurately represent the stationary phase characteristics of such cells in nature. The interaction between *E. coli* host cells and T4 bacteriophage is known to change in stationary phase. This study uses a standard plaque assay to observe the effects of *E. coli* ZK126 culture age on the ability of T4 bacteriophage to form infective centers. Cells grown for 2-9 days were subsequently infected with T4 bacteriophage at a multiplicity of 1 and plated with or without chloroform lysis at 60 and 120 minutes post infection. Cultures infected for 4, 5 and 9 days produced intact, unreleased phage progeny by 60 minutes. These were released in 4 and 5 day-old cultures by 120 minutes. Cultures incubated for longer and shorter periods did not produce or release high concentrations of assembled phage at either time point. When the supernatants of 2 day-old and 4 day-old cultures were swapped, it was found that cells that formerly showed little viral progeny production gained the ability to assemble, but not autonomously release, over 1,000 viral particles per infected *E. coli* cell. The 4 day-old cultures that formerly produced and released viral particles lost the ability to autonomously release viral progeny when swapped into 2 day-old cell supernatant. These results suggest that physiological changes in stationary phase *E. coli* ZK126 affect the efficacy of T4 bacteriophage infection and highlight the need for more stationary phase studies representing the actual events that occur in nature.

Escherichia coli is the most extensively studied bacterium in microbiology. However, the majority of *E. coli* studies are performed using fresh or log phase cultures and may not accurately represent the characteristics of such cells in nature. Rather, bacteria in nature often survive in conditions that lead to stationary phase, where there is no net increase or decrease in cell number (6). Stationary phase may be caused by either the depletion of an essential nutrient in the culture medium or the accumulation of a waste product to an inhibitory level which halts exponential growth (6). In order to cope with such conditions, the cell physiology may change. For example, studies have shown that while stationary phase cells often exhibit decreased metabolism and general biosynthetic processes, they are also capable of select protein overproduction (8). Another characteristic of stationary phase is the increased rate of deleterious genomic mutations (4). These dramatic physiological differences lead to an interest in performing studies on stationary phase *E. coli*, as well the relationship between stationary phase *E. coli* and other organisms.

T4 bacteriophage is a commonly used and highly studied virus that infects *E. coli*. However, the characteristics of T4 infection have also been shown to change in slowly growing or stationary phase cells. For example, in log phase cells, virions are commonly released in a lytic burst, but in stationary phase cells, this lysis is delayed as pseudolysogens are found remaining for longer times within the intact host (5). In fact, unpublished observations by Kutter *et al.* illustrate that the ability of T4 to produce infective centers when mixed with stationary phase *E. coli* decreases with the increasing age of cell culture (www.evergreen.edu/phage/). However, as the physiology of *E. coli* continuously changes as the culture ages, there is a possibility that infective center production may increase or decrease at different time points. Further research to determine whether such an evolving bacteria-to-phage relationship exists may provide useful insights on their real interaction in nature.

MATERIALS AND METHODS

Bacteria Strain and Culture Maintenance. *Escherichia coli* strain ZK126 was obtained as a generous gift from the *E. coli* Genetic Stock Center (New Haven, USA) and was used in all samples as the infection host. This *E. coli* is a derivative of the common K-12 strain but is further characterized by the chromosomal markers Δ(*argF-lac)169*, λ^-, IN(*rrnD-rrnE*)1, *rph-1* and *tnaA5*. Stationary phase cultures, containing 2.3×10^7 cells, were grown in 25 mL M9 media, made using 1x M9 salts (1), 0.02% casamino acids, 0.4% glycerol, 0.1 mM $CaCl_2 \cdot 2H_2O$, and 0.1 mM $MgSO_4 \cdot 7H_2O$. All cultures were incubated at 37°C with aeration. The correlation between cell concentration and turbidity (OD_{600}) was obtained by measuring the turbidity of a culture using a spectrophotometer and dilution plating on LB (Tryptone 10 g, Yeast Extract 5 g, NaCl 10 g to 1 L

of water, pH adjusted to 7.0) with 1.5% agar every hour for 9 hours. Correlation was found to be [Actual cell/mL] = 66.258 x OD_{600}; however, the actual trend of the standard curve appears to be non-linear due to forward scattering (Fig. 1). After this standardization step, all culture concentrations were estimated by measuring the OD_{600} and applying the above equation. Because of forward scattering, the bacterial concentration curve was overestimated, resulting in overestimated cell concentration for given OD measurements. As a result, when cells were infected with an MOI of 1, the actual MOI was slightly more than 1. All fresh ZK126 cultures used for the plaque assay were inoculated in LB media. The turbidity of the cultures was also checked from 18 to 25 hours. Our results indicate that turbidity at 18 hours post-inoculation and subsequent hours leveled at approximately OD_{600} = 3.7, indicating a cell concentration of $2x10^9$ cells/mL. According to Stent (9), stationary phase bacteria reach concentrations of 10^9 cells/mL, which is in accordance with our experimental result. Thus, it is concluded that a starting culture inoculated with $9x10^6$ cells/mL in M9 media will reach stationary phase by 18 hours. As a result, all of our subsequent experiments on stationary phase *E. coli* were performed on cultures that were at least 18 hours old.

Preparation of T4 Viral Stock. Bacteriophage T4 was obtained as a generous gift from the UBC Department of Microbiology and Immunology (Vancouver, Canada). A viral lysate stock was prepared by infecting a 25 ml culture of log phase *E. coli* ZK126 with 10 µl of a $3x10^{10}$ pfu/ml bacteriophage T4 solution. After 4 hours the majority of cells had lysed and cell debris was pelleted using centrifugation to obtain the free phage in the supernatant. Viral titre was determined to be 2.82 $x10^{10}$ pfu/mL using a common plaque assay procedure as described by Stent (9). The stock viral lysate was transferred to a sterile test tube and stored at 4°C throughout the course of this study.

Monitoring T4 infection of stationary phase *E. coli.* Actual cell concentrations of stationary phase *E. coli* cultures were first determined by performing OD_{600} measurement (age of culture = 2, 3, 4, 5, 6, 7, 8, and 9 days). All cultures were infected with MOI = 1. Infected cultures were incubated in 37°C shaking water bath for 60 and 120 minutes. At each time point, 200 µL of sample was treated with an equal volume of chloroform, of which 100 µL was further diluted and plated using the plaque assay mentioned above. For each time point, 100 µL of sample was also assayed directly, without treatment by chloroform. Sample dilutions and 300 µL of log phase *E. coli* ZK126 grown in LB were added to 3 mL of LB with 0.75% agar and plated onto LB with 1.5% agar. Plates were incubated at 37°C for 24 hours and the number of plaques formed was recorded.

Determining the effect of media of different ages on T4 infectivity in stationary phase *E. coli.* Three conditions were set up for this experiment: 1) no media exchange, 2) media exchange and 3) mechanical control. For the no media exchange condition, 2-day and 4-day old cultures were used as is. Media exchange was achieved by centrifuging cultures of 2-day and 4-day old cells at 7000 x g for 10 minutes and resuspending the 2-day old pellets in 4-day old media and vice-versa. The resuspended cells were incubated at 37°C with shaking for 30 minutes to allow any changes in protein induction in the cells to occur. As mechanical controls, the 2-day and 4-day old cultures were first centrifuged, and then resuspended in their original media to account for any effect due to the process of switching media. Cells from all conditions were infected with T4 bacteriophage at an MOI of 1 by using the previously described procedure for the infection assay. The mechanical control conditions were sampled using the plaque assay described for 60 minutes post-infection. Both the no media exchange and media exchange conditions were sampled 120 minutes post-infection.

Pulsed Field Gel Electrophoresis. Stationary phase cultures of *E. coli* ZK126 were grown in M9 for 2, 5, 6 and 9 days, and infected with T4 bacteriophage at an MOI of 1 after measuring OD. A log phase culture of *E. coli* ZK126 grown in LB + glucose was also infected with T4 at an MOI of 1 as a positive control. A culture of uninfected stationary phase *E. coli* ZK126 grown in M9 for 2 days was used as a negative control. Infections were incubated with aeration for 30 minutes at 37˚C before the cells were centrifuged at 10,000 x *g* for 5 minutes at 20˚C. The cells were washed once using Cell Suspension Buffer (10 mM Tris, pH 7.2, 20 mM NaCl, 50 mM EDTA), centrifuged at 10,000 x *g* for another 5 minutes at 20˚C, and then re-suspended in Cell Suspension Buffer. One percent agarose plugs were prepared using $1x10^8$ cells/100 µL plug. The plugs were then incubated in 400 µL Lysozyme Buffer (10 mM Tris, pH 7.2, 50 mM NaCl, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine, 1 mg/mL lysozyme added just before use) per plug for 1 hour at 37˚C without agitation. Following lysozyme treatment, the plugs were incubated in 400 µL Proteinase K Reaction Buffer (100 mM EDTA, pH 8.0, 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine, 1 mg/mL Proteinase K added just before use) per plug overnight at 50˚C without agitation. Next, the plugs were washed using 1 mL Wash Buffer (20 mM Tris, pH 8.0, 50 mM EDTA) 4 times, with a 30 minute incubation and gentle agitation for each wash. The plugs were stored in Wash Buffer at 4˚C for 1 week before PFGE. A 1.0% agarose gel was prepared for PFGE using 0.5x TBE Buffer (45 mM Tris, 45 mM borate, 1.0 mM EDTA, pH 8.3) and the plugs were loaded onto the gel, along with CHEF DNA Size Markers Yeast Chromosomal (Bio-Rad, CA). The gel was run at a gradient of 6 V/cm, an included angle of 120˚, an initial switch time of 1 minute with a linear ramp to a final switch time of 2 minutes for 24 hours using a CHEF-DR® Pulsed Field Electrophoresis System (Bio-Rad, CA). Following PFGE, the gel was stained with 0.5 µg/mL ethidium bromide in water for 1 hour, then the DNA was visualized using an AlphaImager 2000 Documentation and Analysis System (Alpha Innotech Corporation, CA). During the first attempt to visualize the DNA, the bands faded within 5 minutes. As a result, the gel was re-stained for another 30 minutes and a picture of the DNA was taken within a few minutes of visualization.

RESULTS

Formation of T4 infective centers in stationary phase *E. coli* ZK126 change with culture age. This study was designed to observe the different interactions between *E. coli* ZK126 and T4 bacteriophage as the age of the *E. coli* culture increases. Firstly, we tested the ability of T4 to produce infective centers upon infection of *E. coli* cultures aged 2-9 days, in order to re-create the observations of Kutter *et al.* (www.evergreen.edu/phage/). Figure 2 shows plaque (or infective center) formation in stationary phase *E. coli* ZK126 60 minutes post infection. Samples not treated with chloroform all had log % values less than 2 except the 3-day old sample. A log % value of 2 means 100% of virus inoculated resulted in a plaque forming unit. This indicates that, except for the 3-day old sample, after infection some stationary phase *E. coli* did not form infective centers. It is expected that all samples not treated with chloroform will produce a value equal or less than 2 if there is no burst and a value larger than 2 if there is a burst and release of progeny.

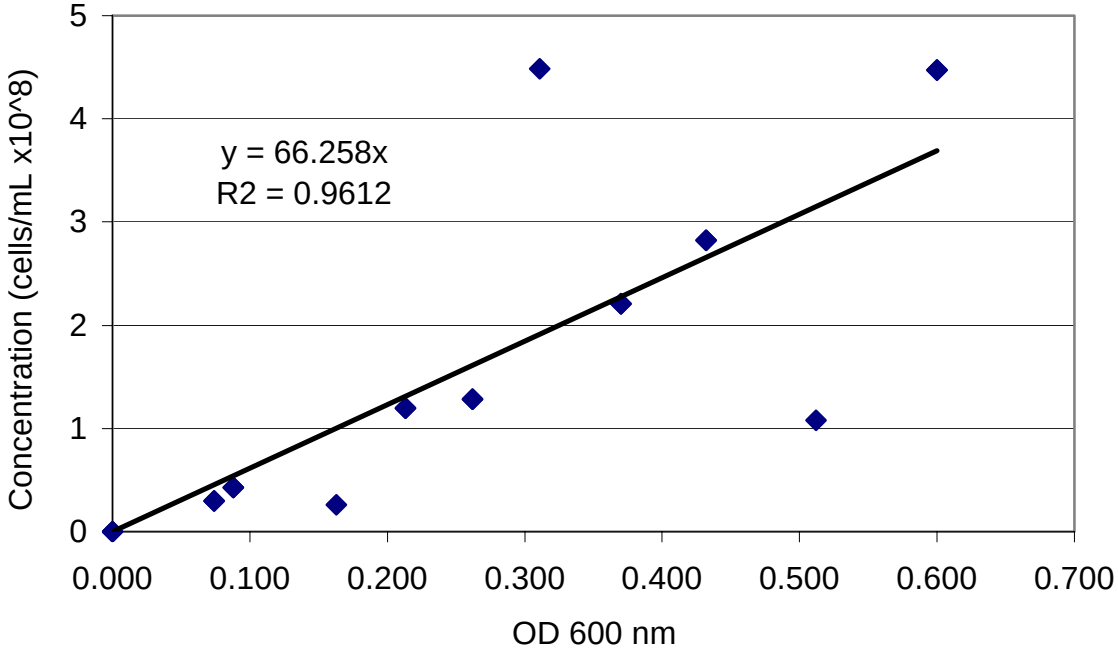

Figure 1. Concentration conversion standard curve for *E. coli* ZK126 grown in M9 media.

Samples treated with chloroform showed a 3,000-fold increase in progeny phages formed in 3- to 5-day old samples, as indicated by a log % value larger 2. However, progeny production dropped drastically in the 2-, 6- to 8-day old samples. In the 9-day old sample, the progeny production resumed and became more effective by 20,000-fold. These fully formed but unreleased phage particles were released by the chloroform treatment.

Comparing the two treatment methods at 60 minutes it can be concluded that there was extremely low progeny production as well as infective center formation in the 2-day old sample even at 60 minutes after infection. Infective center formation and progeny production rose in the 4- and 5-day old samples. This means that not only was infective center formation increased in these samples compared to a 2-day old sample, but also that there were many progeny phage trapped inside the cells, as indicated by the chloroform treated sample resulting in 10-fold more infective centers than the non-treated sample. However, for the 6- to 8-day old cultures, although some infective centers were produced, the progenies were unassembled within the cells, as indicated by the chloroform treated samples data. In the 9-day old culture, infective centers were formed, and in addition, the progenies were assembled but unreleased inside the cell.

Figure 3 shows infective center formation in stationary phase *E. coli* 120 minutes post-infection. All samples that were not treated with chloroform have a log % value less than 2, except for the 4- and 5-day old cultures. This indicates that, except for the 4- and 5-day old sample, infective centers could not form well in all other aged cultures. In the 9-day old sample, the formation of infective centers slightly increased. This indicates that the burst (or lytic cycle) did not occur in any sample except for the 4- and 5-day old samples.

Both the 60 and 120 minutes infections that underwent chloroform treatment show a very similar trend. Again, little or no progeny was formed in the cell in the 2-day old sample and an excessive amount of progeny was detected in the 3-, 4-, 5- and 9-day old samples. The amount of progeny production dropped significantly in the 6-, 7- and 8-day old samples.

Comparing the two treatment methods in 120 minutes, it can be concluded that infective centers did not form well in the 2-day old sample and a significant amount of viral progenies was not produced. In the 3-day old sample, the cells did not go through the lytic cycle yet, but there seemed to be a large amount of progeny viruses unreleased inside the cells. In the 4- and 5-day old samples, both the chloroform treated and non-treated samples had values that approximate each other. In the 6-, 7- and 8-day old cultures, the progeny production again decreased drastically and there was decreased formation of infective centers. Phages may have existed in DNA form inside the cells. It was noted that this DNA form could not be revived to form infective centers even when the cells were plated on rich

media. In the 9-day old sample, again there seemed to be a slight increase in progeny production in the cells but not in the ability to burst.

Comparing the trend for infection for the 60 and 120 minutes samples, the only difference seems to be a lower value in infective centers for the 3-day old sample not treated with chloroform, a higher value for the chloroform treated 3-day old sample, and a general increase in magnitude in infective centers for the 4-, 5- and 9-day old samples treated with chloroform. This is significant because it means that in these cultures, the cells burst (or underwent a lytic cycle) which caused the number of free progeny in solution to increase 100-fold more than the original input amount of virus. Therefore, in the 4- and 5-day old samples, burst occurred between 60 to 120 minutes post-infection.

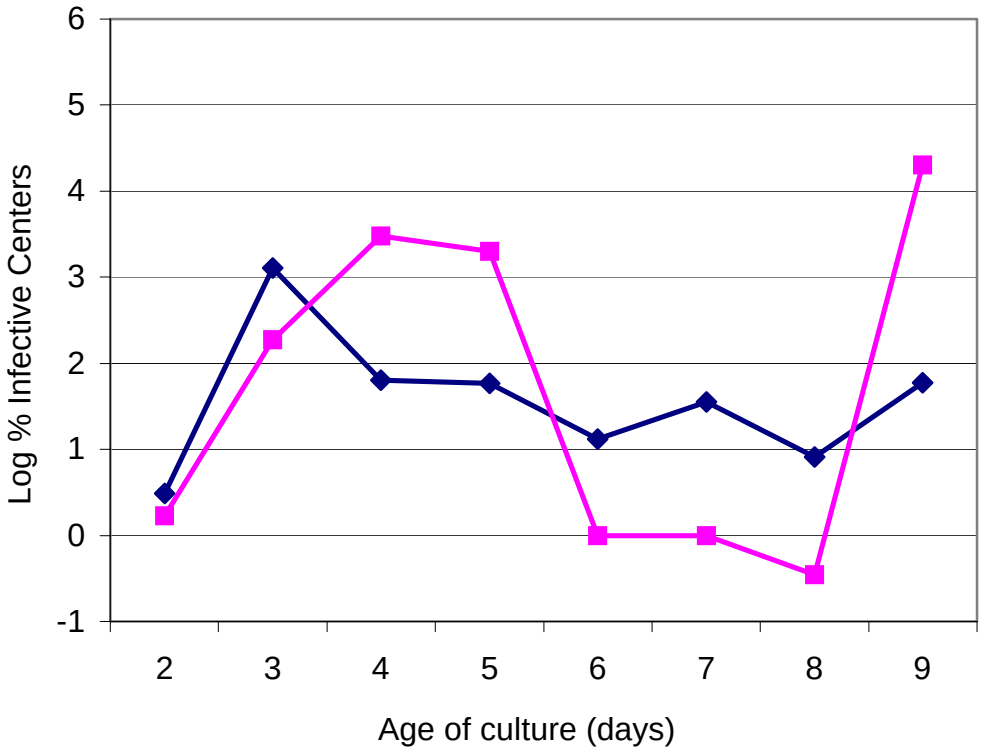

Figure 2. Percent infective centre formation in stationary phase *E. coli* ZK126 of different ages after 60 minutes. Results are expressed as log % of plaque forming units per bacterial cells plated. Diamonds represent samples not treated with chloroform and squares represent chloroform treated samples.

Extrinsic media factors are important for T4 phage infectivity in stationary phase *E. coli*. To confirm that the mechanical procedures of swapping media, such as centrifugation and cell resuspension, did not significantly affect measurements of the percentage of infective centers, we included a mechanical control. We observed no significant changes in infective centers between the mechanical controls and the no media exchange samples (results not shown on graphs).

Figure 4 shows the log percentage of infective centers formed in 2- and 4-day old *E. coli* plated at 120 minutes post infection, with and without media switching. From this graph, we noticed a 40,000-fold increase in the percent of infective centers formed when 2-day old cells are switched into 4-day old supernatant (from a 4-day old culture), compared to without media exchange. In contrast, there was a 25,000-fold decrease in the percent of infective centers formed when 4-day old cells were switched into 2-day old supernatant.

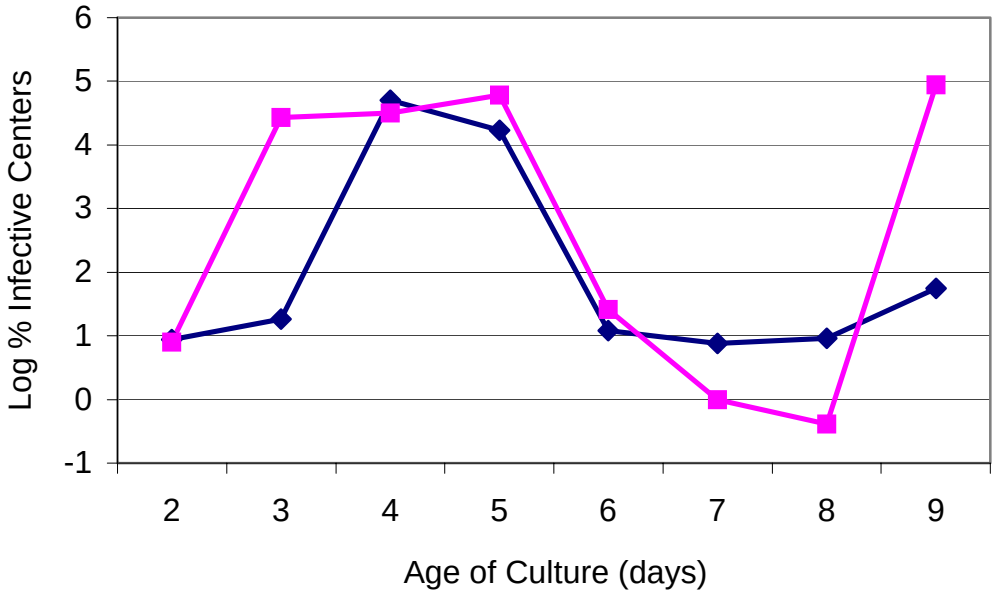

Figure 3. Percent infective centre formation in stationary phase *E. coli* ZK126 of different ages after 120 minutes. Results are expressed as log % of plaque forming units per bacterial cells plated. Diamonds represent samples not treated with chloroform and squares represent chloroform treated samples.

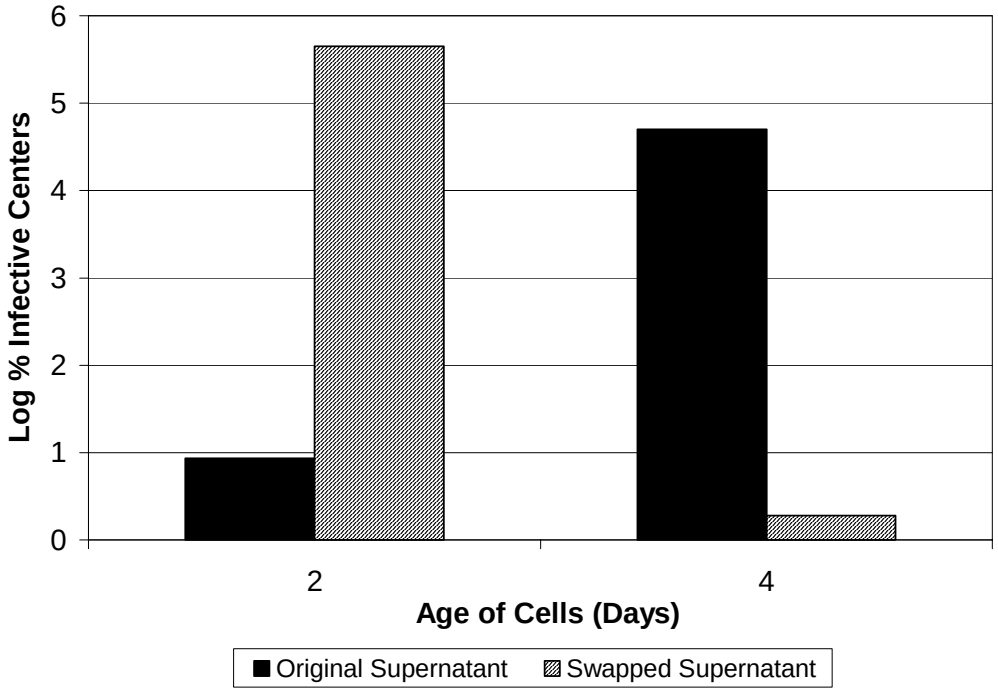

Figure 4. The effect of media switching on T4 bacteriophage infectivity in stationary phase *E. coli* ZK126 plated at 120 minutes post infection.

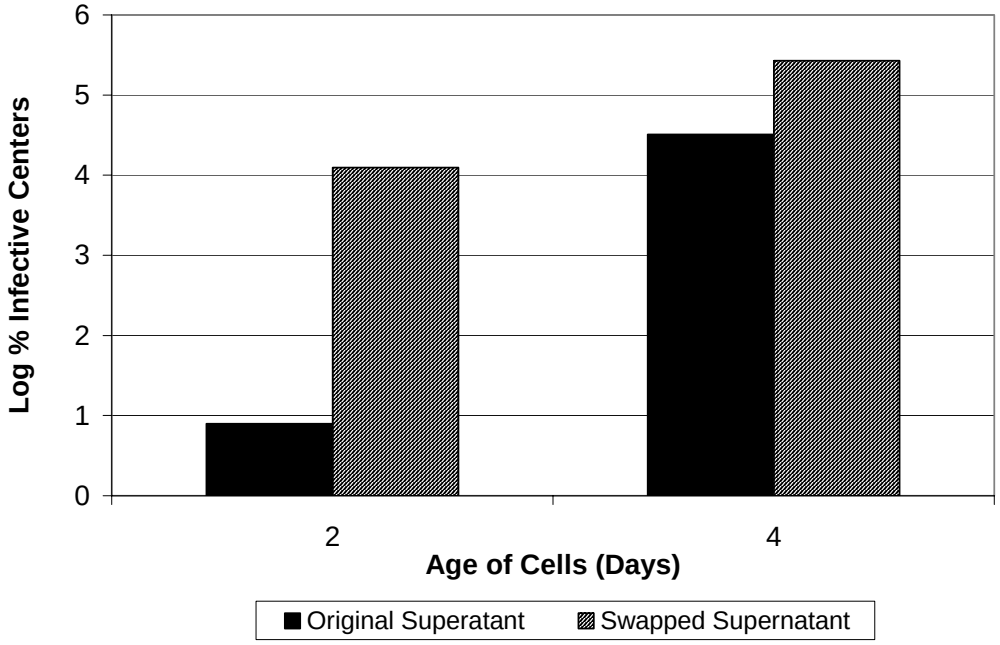

Figure 5. The effect of media switching on T4 bacteriophage infectivity in stationary phase *E. coli* ZK126 lysed with chloroform and plated 120 minutes post infection.

Figure 5 is similar to figure 4, with the exception that cells were treated with chloroform prior to plating in the plaque assay (thereby artificially releasing assembled phage). There was a 1,200-fold increase in the percent of infective center formation when 2-day old cells were swapped into 4-day old supernatant, which is a significant change in comparison to a 40-fold increase in the percent of infective centers when 4-day old cells were placed in 2-day old supernatant.

In addition, if the results for the no-chloroform treatment and the chloroform treatment are compared, there was a significant decrease in the amount of progeny autonomously released by the 4-day old cells not treated with chloroform when switched into a 2-day old supernatant. The 4-day old cells produced less than 10% infective centers without chloroform, but subsequently produced more than 100,000% infective centers after chloroform lysis. This result was unrealistically high, indicating some dilution or plating error might have occurred. In contrast, comparing between the 4-day old cells in 4-day old media and 2-day old media, there was only a slight change in the number of infective centers after artificial lysis with chloroform. The increased amount of infective centers formed when 2-day old cells were switched into 4-day old media seemed to be higher in the non-chloroform treated samples compared to the chloroform treated samples.

150 kb DNA is found in 9-day old *E. coli* but not in 2-day old *E. coli* infected with T4 bacteriophage. To further examine the different interactions of T4 with *E. coli* cultures of various ages, pulse field gel electrophoresis (PFGE) was used to visualize the existence or non-existence of T4 DNA within the host. The T4 bacteriophage infection cycle begins when the virus attaches itself to the core polysaccharide of the *E. coli* cell wall (6). DNA absorption speed is related to host cell density, whereby a virus introduced to a host cell concentration of 10^8 cells/ml should be 90% adsorbed within ten minutes (9). Unpublished observations by Kutter *et al*. suggested that such high adsorption efficiencies remain true in stationary phase cells of varying ages (www.evergreen.edu/phage/). However, it is uncertain whether this absorption efficiency translates into the maintenance of viral DNA within the host and therefore was studied here using PFGE. Cultures of *E. coli* ZK126 were grown for 2, 5, 6, and 9 days, infected and incubated with T4 bacteriophage at an MOI of 1, washed free of unadsorbed phage and prepared for

PFGE. Uninfected *E. coli* grown for 2 days was used as a negative control, while infected log phase *E. coli* was used as a positive control. A picture of the PFGE gel is shown in figure 6. Lane 4 contains DNA size markers ranging from 0.2 to 2.2 Mb. In the positive control sample (lane 1), a 150 kb band, corresponding to the size of the T4 genome, is visible. The 150 kb band is also observed in the infected 9-day old sample (lane 7). In contrast, there is no 150 kb band in the uninfected control (lane 2) or the infected 2-day old sample (lane 3). The presence of a 150 kb band in the infected 5-day old sample (lane 6) or the infected 6-day old sample (lane 7) cannot be confirmed due to smearing of the DNA. Also, more DNA ran in these lanes than the others, as determined by the relative intensity of the DNA left in the sample wells. The *E. coli* genome is approximately 4 Mb, and is visible in all the lanes, except lane 4, as unresolved DNA in the sample wells. A 1.3 Mb band is also observed in all lanes (except lane 4). In addition, faint 1.0 and 2.3 Mb bands are visible in the uninfected control, the infected 2-day old sample and the infected 9-day old sample. Finally, the white streak through the last three lanes at approximately 610 kb corresponds to a fracture in the gel that occurred while taking the picture.

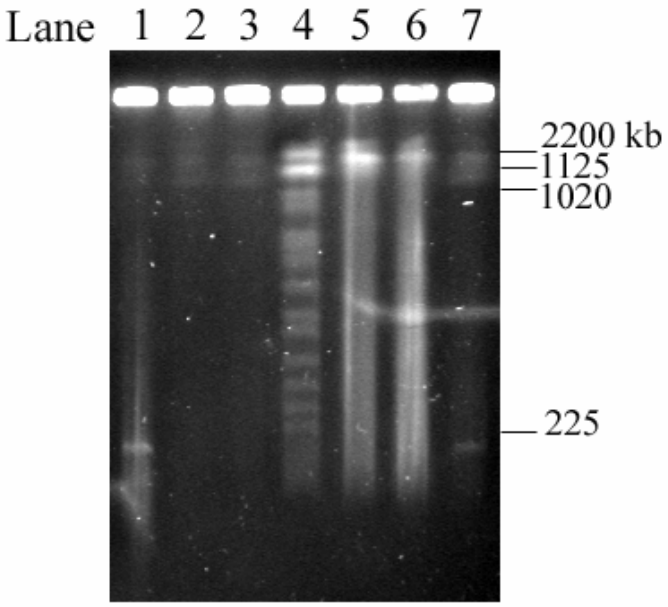

Figure 6. PFGE gel with ethidium bromide fluorescence of total DNA from *E. coli* cultures of different ages infected with T4 bacteriophage. The samples are infected log phase cells (lane 1), uninfected 2-day old cells (lane 2), infected 2-day old cells (lane 3), 0.2 to 2.2 Mb DNA size markers (lane 4), infected 5-day old cells (lane 5), infected 6-day old cells (lane 6) and infected 9-day old cells (lane 7).

DISCUSSION

In this experiment, a trend was observed when stationary phase *E. coli* cultures of different ages were infected with T4 bacteriophage:

(1) 2-days old: T4 in infected cells could not form infective centers nor produce progeny.
(2) 4- to 5-days old: T4 in infected cells produced progeny at the amount equivalent to one lytic cycle (approximately 100-fold). The lytic burst occurred at some time between 60 to 120 minutes post-infection to release viral progenies.
(3) 6- to 8-days old: The susceptibility of cells to T4 infective center formation and production of viral progeny decreased sharply to near zero levels.
(4) 9-days old: Infected cells were able to produce progenies but these were not released (or burst).

The observations from this study suggest that as *E. coli* cells age in stationary phase, their physiology and susceptibility to T4 progeny production and lysis changes. At days 4-5, they are more susceptible, while at days 6, 7 and 8 they are barely susceptible. This change in susceptibility seems to occur in a low-high-low-high manner from day 2 to day 9 and may be cyclical. Research that extends further into stationary phase is needed to test this hypothesis.

As it stands, the changing susceptibilities of stationary phase *E. coli* to T4 infective center formation seen in this experiment may be explained by several mechanisms. For example, the changes in the infection susceptibility of *E. coli* may be explained by a phenomenon known as hypervariability in stationary phase cells. It has been proven that different ages of stationary phase cultures are genetically different (11). In addition, a study by Yeiser *et al.* (10) suggests that the ability of a cell to acquire advantageous genes and survive in stationary phase media depends on its ability to mutate. This ability is called the GASP phenotype, which is caused by the hypervariability of the *E. coli* cells (10). Our study suggests that changes in *E. coli* phenotype may be occurring in a cyclic pattern, thereby causing physiological changes that alter the cell from having a low susceptibility to T4 infection to high susceptibility to T4 infection. Furthermore, this change in susceptibility to T4 bacteriophage infection and progeny formation seems to make sense when considering the state of *E. coli* in the environment. In nature it may be likely that *E. coli* exist in alternating states of susceptibility to viruses as part of a natural equilibrium. Resistance mechanisms may prevent *E. coli* from being constantly susceptible; a state that may be disadvantageous to the bacterial population. Hypervariability as described by Yeiser *et al*. (10) may be one such natural resistance mechanism of *E. coli* against T4 bacteriophage that helps to maintain this equilibrium.

Another hypothesis for the cell's cyclic susceptibility to progeny formation may be due to the changing availability of carbon. As cells consume surrounding nutrients, carbon sources may eventually become exhausted, but then later released when a portion of cells die due to starvation. The intracellular substances released by these dying cells become available for use by other cells in the culture. In a study by Hadas *et al.* (3), it was observed that the increase in nutrients benefited the growth of bacterial cells and thereby increases the production and release of viral progeny when those cells become infected. Perhaps at later stages of stationary phase, the increased amount of cell death leads to the re-availability of labile carbon compounds and other nutrients that support the growth of some *E. coli*, which in turn allows phage to form progeny. Also, since energy is required for viral particles to form, respiration of the newly available labile carbon compounds may support this energy requirement.

A hypothesis to explain the low levels of autonomous viral progeny released in some samples involves a phage cycle known as pseudolysogeny. It has been shown that when T4 infects slowly growing bacterial cells, the phage and host may co-exist in an unstable fashion where lytic bursts are significantly delayed (5). This may explain our observation of accumulated phage particles within stationary phase cells that were only released after artificial lysis by chloroform.

Our observations also included some anomalies. For example, the 3-day old sample showed greater infective center formation without chloroform artificial lysis than with chloroform treatment. This is not likely because any non-treated sample should not have a value larger than log % = 2 if there was no lytic burst. If there was a burst, the chloroform treated sample would likely have a value higher than the non-treated sample. Therefore, in the graphs, we expect to see the chloroform treated sample's trend line to be lower than the non-treated sample's trend line. However, this is not seen for every time point.

The limitations of this study may have contributed to the observed anomaly. In particular, it should be noted that the experimental results in this study are based on a single replicate, and therefore, conclusions drawn from them should be made conservatively. Also, prior to compiling the final data set used in the analysis, data had been obtained but not used for 2- and 9-day old samples. These values were rejected on the basis that they were unnaturally high because the number of T4 phages far exceeded the volume capacity of available bacteria, and were therefore highly suspicious in terms of technical error. Reported results shown here are based on the most recently acquired data set, after samples were reprocessed and replated.

Another limitation of this assay lies in the varying susceptibility of infected *E. coli* to lysis by chloroform. Chloroform lysis of bacterial cells depends on the condition of the bacterial cell wall. Upon T4 bacteriophage infection, viral lysozymes are produced that weaken the bacterial cell wall and increase the cell's susceptibility to artifical lysis by chloroform (9). Therefore, if the ability of T4 to produce lysozyme in *E. coli* of various stationary phases changes, the susceptibility of these cells to chloroform lysis may also change. It is possible that some of the *E. coli* samples contained more assembled but unreleased phage that could not be artificially released by chloroform as intended. This adds another variable to our results that necessitates a conservative approach to analysis.

Finally, this part of our experiment was performed in order to confirm Kutter's unpublished observations that when T4 bacteriophage infect *E. coli* cultures of increasing stationary phase ages, infective center formation decreases at first and then increases slightly to 10% in 4- to 10-day old cultures (www.evergreen.edu/phage/). Kutter called the phenomenon of decreased infective center formation "hibernation." However, the exact procedures used in the previous experiment are unknown and therefore our experimental designs likely differ. The results of Kutter's and our experiment may not be directly comparable. In fact, a 10% increase in the ability to form infective centers was not observed in our experiment. The 2-day old sample may be considered to be in a "hibernation" state whereas the 4- and 5- day old samples may be in a state of revival and able to form infective

centers. This identification of possible hibernation and revived infective center formation states led to the subsequent stage of this investigation whereby the cause for this physiological change was examined.

The cell pellets of 2- and 4-day old *E. coli* cultures were isolated and swapped into supernatants corresponding to 4- and 2-day old cultures, respectively. It should be noted that the observations of the media swap experiment are limited to those related to changes in early protein induction pathways because we incubated the cell suspension for only 30 minutes after media switching. According to a joint study done by Silico Insights and Ashni Naturaceuticals (www.silicoinsights.com), it takes 20 minutes for early up-regulated gene expression and more than 1 hour for late up-regulated gene expression. Therefore, a 30 minute incubation would only allow for early protein induction to occur with little to no change in the physiology of cells, such as changes in cell membrane structure.

Since we notice that 2-day old cells allow increased infective center formation after being switched into 4-day old supernatant, there are likely factors present in the 4-day old supernatant that contribute to this increase. One of the possibilities is that at later stages of stationary phase, the increased rate of cell death leads to the release and accumulation of labile carbon compounds and other nutrients, such as amino acids, into the supernatant that support the metabolism of infected *E. coli*, which in turn allows phage to form progenies.

Conversely, it is also possible that there are factors in the 2-day old supernatant which inhibit the efficient release of progeny in 4-day old cells. Unpublished observations by Kutter *et al.* suggest that during an infection, 99% of phages are adsorbed by stationary phase cells (www.evergreen.edu/phage/). However, in this experiment, even though most cells were infected and produced the expected high amount of progeny in the non-chloroform treated samples, few infective centers were formed. Factors in the 2-day old supernatant may affect the expression of certain lysozymes that are required for efficient progeny release, and alteration in their expression efficiency may lead to a change in the ability of the infected cells to form infective centers.

One inhibitor that may be responsible for the less than 100% infective centers seen in 2-day old cells in 2-day old supernatant could be methyl α-D-glucoside, which is a competitive inhibitor of glucose uptake (3). The accumulation of this compound prevents the use of glucose that is present. Since the growth of viral progeny is directly related to bacterial growth, which is in turn altered by the presence of glucose, the accumulation of such a compound into the media can lead to a decrease in the number of fully formed phage (3).

Another interesting observation is the difference in patterns between the no-chloroform and chloroform treatment conditions. The difference between the two conditions sheds some light behind the changes noted after media switching. For example, there is only a small difference between 4-day old, non-switched, chloroform treated cells and non-treated cells. This is because all progenies are already released after 120 minutes before chloroform treatment. Also, there is a significant decrease in the amount of infective centers in the 4-day old cells after being switched into a 2-day old supernatant without artificial lysis. However, when these cells were lysed with chloroform, an increase in infective centers was observed. One reason for this may be that 4-day old cells swapped into 2-day old media lose their susceptibility to autonomous T4 bacteriophage progeny release. Since T4 can form pseudolysogens, which inhibit cell lysis, it is possible that the phage use this mechanism to optimize phage development in slowly growing cells (5).

There is no dramatic decrease in the percent infective centre formation in 4-day old cells (chloroform treated) with and without media switching. It is possible that these cells are physiologically different than 2-day old cells. For instance, adjusting to a stationary phase GASP phenotype enables *E. coli* in later stationary phase to grow better (4). In comparison, 2-day old cells may not have developed such characteristics required to overcome the difficulties imposed on them by the stationary phase environment (12). Therefore, when 4-day old cells are switched into 2-day old supernatant prior to infection, they still possess the characteristics or nutrients required for viral progeny formation.

Interestingly, the increased amount of infective center formation in 2-day old cells after media switching seems to be higher in the no treatment samples compared to the chloroform treated samples. It is possible that not all of the progeny are being formed into full particles that are able to infect cells. Instead, the phage might exist in DNA form or in the form of incomplete particles, which may account for our observation of a lower percent of infective centers in chloroform treated samples for the 2-day old cells. Incomplete particles or DNA cannot form infective centers when artificially released from the host cell.

To study the ability of T4 bacteriophage to inject its DNA into stationary phase *E. coli* 30 minutes after infection, the presence of viral DNA in host cells was investigated. A 150 kb DNA was found in both log phase and 9-day old *E. coli* infected with T4. This size of DNA corresponds to the known size of the T4 genome and the absence of a 150 kb DNA in uninfected cells strongly suggests that this is T4 DNA. However, to confirm that it is T4 DNA, the DNA band will need to be checked in a future study by southern blotting and hybridization using an anti-T4 DNA probe. The presence of T4 DNA in infected 9-day old *E. coli* confirms the results seen from the infection assay where the accumulation of fully assembled, but unreleased phage was observed in infected cells after 60 and 120

minutes. Surprisingly, no T4 DNA was detected in infected 2-day old *E. coli*. There are three possibilities that may account for this observation. The most likely reason for this may be that viral DNA in infected cells after 30 minutes is in concatomeric form, which can be up to 20 times as large as a single phage genome, or 3 Mb, because T4 replicates by rolling circle replication (7). Such large concatomers would not have resolved well in the PFGE from the 4 Mb host genome, and thus, would remain in the band present in the sample wells. This possibility suggests that 2-day old cells could have viral DNA, although not in a 150 kb form. To confirm the presence of concatomers, the gel should be run longer in a future study to try to resolve the large viral DNA from host DNA. However, if no concatomers are present, other reasons must explain the lack of 150 kb DNA in infected 2-day old cells. After taking into account that the T4 genome is 27 times smaller than the *E. coli* genome, a minimum of 0.01 μg of DNA can be detected on an agarose gel, 0.01 μg of DNA corresponds to approximately $1x10^5$ cells, and thus, T4 DNA should have been detected even if each cell only had one viral genome. Either the adsorption and injection of T4 DNA does not occur to a detectable level or T4 DNA exists as a non-detectable form in 2-day old cells. The possibility that adsorption and injection does not occur in most 2-day old cells is unlikely, as little free phage was observed in infected 2-day old samples in the infection assay. However, it is possible that after T4 adsorption and DNA injection into 2-day old cells, the T4 DNA transforms into a form not detectable by ethidium bromide. This may account for the 1% infective center formation seen in the infection assay, which differs from the 100% that was expected. Also, the undetectable form of phage DNA may be related to the "hibernation" effect that Kutter *et al.* observed (www.evergreen.edu/phage/). Alternatively, the T4 DNA may have been degraded upon injection into the cell if 2-day old, but not 9-day old cells, have a mechanism for degrading gp2, the viral protein that protects viral DNA from degradation (7). In summary, T4 accumulates as fully assembled particles in 9-day old *E. coli*, but does not exist as free phage or as free viral DNA inside infected 2-day old cultures.

The presence of T4 DNA in infected 5- and 6-day old *E. coli* could not be confirmed due to DNA smearing in the PFGE gel. Some DNA smearing was also observed in the infected log phase culture. DNA smearing in PFGE gels is usually due to excess cell concentrations or DNA degradation (2). The amount of cells used to prepare the PFGE samples in this study, $1x10^9$ cells/mL, was well within the recommended concentrations of $5x10^8$ to $2.5x10^9$ cells/mL (2). In addition, an equal amount of cells was used to prepare each sample, therefore, each lane contained the same amount of DNA. Thus, the observed DNA smearing was likely due to DNA degradation of the large *E. coli* genome and not due to an unequal amount of sample in the lanes. Because DNA degradation was only observed in the lanes containing 5- and 6-day old cells and not in the other lanes, degradation must be specific to the age of the cells, as stationary phase cells of different ages are genotypically and phenotypically different from each other (11). Since T4 bacteriophage produces a nuclease to degrade host DNA (7), it is possible that the nuclease is expressed or functions more efficiently in 5- and 6-day old cells whereas in 2- and 9-day old cells, nuclease expression or function might be inhibited in some way by the host. This might account for the DNA smearing seen for 5- and 6-day old cells and not for 2- and 9-day old cells.

Several bands, 1, 1.3 and 2.3 Mb, were found belonging to the host genome in addition to the usual 4 Mb genome found unresolved in the sample wells. A 1.3 Mb DNA was found in all uninfected and infected samples, while the 1 and 2.3 Mb DNA was observed in small amounts in the uninfected, the infected 2- and 9-day old samples. These extra bands may be different forms of chromosomal DNA, which frequently appears as large bands near the top of PFGE gels (2) and are probably not plasmids, as *E. coli* ZK126 is not known to have any plasmids. In addition, because all three sizes of DNA are found in the uninfected culture, these bands are not concatomers from rolling circle T4 replication.

In summary, the current study indicates that (a) different ages of *E. coli* culture in stationary phase causes different effects in T4 progeny product and burst during an infection (b) factors present in the cultures media is at least partly responsible for the change from lack of progeny production and burst to full progeny production and burst from a 2- to 4-day old sample and (c) viral DNA is present in the expected size in an infected 9-day old culture but not in a 2-day old culture.

FUTURE EXPERIMENTS

Further research into the ability of T4 bacteriophage to form infective centers in *E. coli* ZK126 of various culture ages may add to the scope of this study. For example, our observations of infective center formation at 60 minutes post infection showed that cells first lose the ability to autonomous release, and then to form, assembled phage in cultures aged for 4 to 8 days. However, in 9-day old cells, some physiological changes in the cells occurred such that the ability to form assembled but unreleased viral progeny was recovered. It would be useful to continue this experiment beyond 9 days to see if the ability to autonomously release viral progeny would also be regained. In our unpublished observations, we noted that the number of infective centers with and without chloroform artificial lysis

reached near zero for 11-day old cultures. Whether the ability to form and release viral progenies follows some cyclical time pattern should be addressed in future research.

Another addition to this study could be to re-observe plaque assay plates beyond the standard 24 hours elapsed between plating and plaque counting. We have suggested that physiological changes experienced by the cell over time may inhibit or allow T4 progeny formation and release. It is possible that cells exhibiting any inhibitory physiological changes require more than 24 hours of growth on the rich media used for plaque assays to revert to a state which allows infective center formation.

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