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The Effect of Culture Age and Extrinsic Factors on T4 Bacteriophage Progeny Production in Recovered Stationary Phase *Escherichia coli* ZK126

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The ability of T4 bacteriophage to maintain a productive infection changes with the growth phase of its host. During stationary phase, *Escherichia coli* specifically up-regulates the expression of specific proteins and down-regulates others. This causes physiological and genetic changes that have a dramatic effect on the susceptibility of *E.coli* to T4 infection. Previous studies have indicated that progeny production changes with the length of time spent in stationary phase by *E.coli*. This study uses a standard plaque assay to observe the effects of *E.coli* ZK126 culture age and recovery on the ability of T4 to produce progeny. Cells grown for 2, 4, and 7 days were recovered for 2 or 24 hours and subsequently infected with T4. Progeny production was not observed in 2-day old cultures. The 4-day old cultures produced the most progeny following a 24h recovery. The 7-day old cultures could only produce progeny if recovered in New media for 2h, which indicates the presence of inhibitors in spent media. These inhibitors are either preventing infection or inducing a pseudolysogenic T4 growth cycle. Further studies examining T4 infection of stationary phase *E.coli* will provide useful information pertaining the interaction that occurs between the virus and these cells.

The environment within which bacteria reside is constantly evolving. Often times, these microorganisms are faced with pressures from their external environment such as a decline in an essential nutrient, namely carbon, or the accumulation of waste products (10). When such pressures exist, exponentially growing cells enter stationary phase. This is often the case when cells are grown in batch culture where there is no addition of nutrients or removal of spent media (10).

As cells enter stationary phase, they continue to remain metabolically active and adapt to their limiting environmental conditions (14). In particular, Gram-negative bacteria, such as *Escherichia coli*, reduce their overall rate of protein synthesis while upregulating the expression of a particular class of proteins known as stationary phase specific σ_s (14). These proteins protect cells from oxidative damage and enable cells to remain viable in stationary phase (14). Stationary phase cells also undergo physiological changes in cell shape (14). Notably, exponential phase cells are rod-shaped while stationary phase cells are more spherical (14). Further, stationary phase cultures typically do not exhibit a net increase or decrease in cell density, however, the culture experiences variable viability, as some cells are able to adjust to limiting conditions while others die off (10). With prolonged incubation, mutations have an increased likelihood of occurring, which can give rise to cells with either a competitive advantage or marked disadvantage (14). Mutations or σ_s genes may even cause stationary phase cultures to release substances into the media (14). Notably, the composition of media derived from prolonged stationary phase cultures may by markedly different from the media of exponentially growing cells (1). Constituents of this spent media have been known to present adverse conditions for cells growing into prolonged stationary phase (1). Subsequently, the effects caused by cells growing in spent media may also affect their ability to recover once transferred into fresh media or when transferred into media supplemented with carbon.

These physiological and genetic adaptations have a dramatic effect on the susceptibility of *E. coli* to bacteriophage T4 infection (3, 7). Bacteriophage T4 is well known for its ability to infect *E. coli* (4). T4 is composed of a DNA-containing head, a tail and a base plate that is attached to both short and long tail fibers (13). T4 attaches to the outer surface of *E. coli* via these tail fibers and subsequently delivers its genome into the host (11). Initial attachment of T4 is weak, thus the shape of stationary host cells may affect this binding (2). The absorption constant (k) of T4 phage to stationary phase *E. coli* has been found to be $k=1 \times 10^{-9}$ ml/minute (1). This can be compared to the standard value $k=2.5 \times 10^{-9}$ ml/minute of log phase *E. coli* cells (1,12). The value of k is comparatively smaller for stationary phase cells because their spherical shape provides a smaller surface area to which phage can absorb (1). The fact that stationary phase cells are smaller reduces the number of collisions between phage and cells such that stationary phase cells will absorb fewer phage and expectantly produce fewer progeny. Following transcription and translation of

phage particles within the host cell, newly assembled phage undergo lysis and release of T4 progeny (5). Normally 100 to 150 phage particles accumulate in the cell by the time lysis occurs (9). For optimal infection, T4 require exponentially growing cells (12). Lysis is often delayed in stationary phase cultures indicating T4 cannot infect as efficiently under stationary conditions (8). Thus, for a productive infection, stationary phase cells may need to first undergo recovery (9).

The ability of T4 to produce and release progeny in stationary phase cells has been shown to decline when compared to exponential phase cells (1, 8). This decline in progeny has been attributed to several factors. The ability of T4 to produce plaques when mixed with stationary phase *E. coli* decreases with the increasing age of cell culture (3). Most notably, varying ages of stationary phase cultures are genotypically different, consequently producing phenotypic changes as well (14). Recent evidence indicates that the ability of T4 to grow in *E. coli* is closely correlated to the physiology of the cell (3, 6). As T4 requires a surface for binding, the shape of the cells may be integral in the infection process. Since stationary phase cells are smaller than log phase cells, there is less surface area available to which phage can bind (1). It has also been suggested that the reduction of susceptibility to infection and progeny release is attributable to cell metabolic poisons or free phage poisons released by cells in spent media (1). Since many factors affect the ability of T4 to infect stationery phase cells, it is beneficial to examine specific culture characteristics including the ability of stationary cells to recover and whether their production of metabolites alters infection. In this study, we specifically examined the ability of *E. coli* ZK126 to recover after various days of growth in stationary phase and whether potential metabolic poisons or inhibitors released by these cells have an impact on their susceptibility to infection by T4.

MATERIALS AND METHODS

Bacteria Strain and Culture Maintenance. *Escherichia coli* strain ZK126 was obtained from the UBC Department of Microbiology and Immunology culture collection (Vancouver, Canada) 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, each inoculated with a loopful of cells, were grown in 25 mL LB media (10 g bacto tryptone, 5 g bacto yeast extract, and 10 g NaCl into 1 L of water) for extended periods of time (2, 4, and 7 days). All cultures were incubated at 37°C with aeration at 180 rpm.

All culture concentrations were estimated by measuring the OD_{600} using a spectrophotometer and applying the following correlation of cell density. Data from Chan *et al* indicates that 1 $OD_{600} = 5.41 \times 10^8$ cells/mL. Also, according to Stent (9), stationary phase bacteria reach concentrations of 10^9 cells/mL, which Chan *et al.* (3) observed to take approximately 18 hours. Consequently, our experiments in *E. coli* were performed on cultures that were at least 18 hours old.

Preparation of T4 Viral Stock. Bacteriophage T4 was also obtained from the UBC Department of Microbiology and Immunology culture collection. A viral lysate stock was prepared by infecting a 25 ml culture of log phase *E. coli* ZK126 with 10 µl of a 3×10^{10} pfu/ml bacteriophage T4 stock solution. Infection occurred overnight, allowing the majority of cells to lyse.

Cell debris was pelleted using centrifugation at 5000 x g for 5 minutes to obtain the free phage in the supernatant. Viral titre was determined to be 1.86×10^8 pfu/mL using the common plaque assay procedure described by Stent (9) and Chan *et al.* (3). The stock viral lysate was transferred to a sterile test tube and stored at 4°C throughout the course of this study.

Monitoring the effects of media at different ages on the recovery of stationary phase *E. coli*. Three media conditions were established for this experiment: 1) old media obtained from cell cultures growing into extended stationary phase (2, 4, and 7 days), 2) old media supplemented with 0.2% carbon (10% glycerol), and 3) fresh LB media. These experimental media have consequently been referred to as: "Old," "Old + C," and "New" media. At various intervals into stationary phase six *E. coli* cultures (age of culture = 2, 4, and 7 days) were separately centrifuged at 10,000 x g for 5 minutes to pellet the cells. Pellets were subsequently resuspended separately into the three experimental media to obtain recovery cultures. Two Old media recovery cultures were achieved for each culture age (2, 4, and 7 day) by resuspending, separately, cell pellets into old media. Two Old + C media recovery cultures were achieved for each culture age by separately resuspending cell pellets into Old media and adding 500 µL of 10% glycerol to each culture. Two New media recovery cultures were achieved for each culture age by separately resuspending cell pellets into New media. One set of cultures was allowed to recover for 2 hours while the other set was left to recover for 24 hours. Cell concentrations for all six cultures were determined by performing OD_{600} measurements at the start of recovery. All six cultures were left to recover while incubated at 37°C at 180 rpm. Following recovery OD_{600} measurements were taken to determine if the cultures had recovered.

T4 infection of stationary phase *E. coli* following recovery. All recovery cultures were infected with an MOI = 10^{-4} and incubated at 37°C at 180 rpm for 60 minutes. A common plaque assay procedure previously described by Chan *et al.* was used (3). The only difference was that 100 µL of indicator cells were used. Plates were incubated at 37°C for 24 hours and the number of plaques formed was recorded. A positive control was also recorded, where exponentially growing *E. coli* was infected for 1 hour at 37°C at 180 rpm and subsequently plated as described above. The amount of Progeny Produced was calculated as a ratio of output T4 (pfu/mL) / input T4 (pfu/mL).

RESULTS

Fig. 1A shows the amount of phage progeny produced in cultures that were grown for 2 days. All of the cultures show a negligible difference in phage progeny produced compare to input phage. The Old + C media sample with a 24h recovery displayed a 4-fold increase in phage production compared to input phage. We assume that this increase is due to experimental error, as we expect that a productive infection with one burst would result in approximately a 100-fold increase in phage production. Note that this is an average value expected for a burst in exponentially-growing cells, thus the actual value in for cells growing in stationary

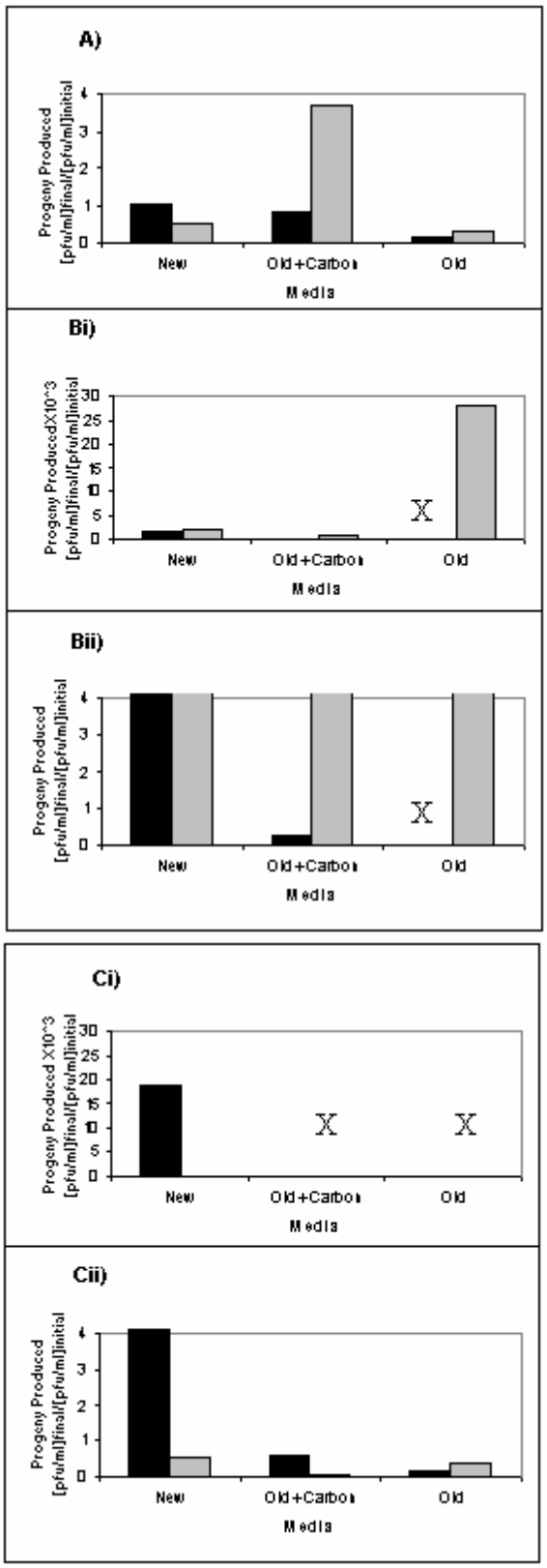

Fig. 1: T4 Bacteriophage progeny produced in *E. coli* ZK126 cultures recovered in various media for 2h or 24h. These cultures were grown in stationary phase for 2 Days (A), 4 Days (Bi and Bii), and 7 Days (Ci and Cii). Black bars represent cultures that recovered for 2h, grey bars represent cultures that recovered for 24h. The X represents no data available for the culture recovered for 2h in Old Media on Day 4.

phase might be much lower (12). Fig. 1Bi shows the amount of progeny produced in cultures that were grown for 4 days. Fig. 1Bii is a reproduction of this graph with a different scale. No data is available for the culture recovered for 2h in Old media. The culture that recovered for 2h in Old + C media is the only culture at Day 4 that showed no increase in phage progeny produced compared to input phage. Cultures recovered in New media for 2h and 24h and Old + C media for 24h exhibited a similar, modest increase in phage progeny produced, ranging from 900-1900 times increased. The culture recovered in Old media for 24h is the only culture that showed a very large increase, with almost 30,000 times more phage progeny produced compared to input phage. In cultures grown for 4 days, all media types showed an increase in phage progeny produced, but only recovery in Old media resulted in an extremely high amount of phage progeny produced. Fig. 1Ci shows the amount of progeny produced in cultures that were grown for 7 days. Fig. 1Cii is a reproduction of this graph with a different scale. Only the culture grown in New media for 2h exhibited an increase in phage progeny produced compared to input phage. This increase is very large, approximately 19,000 times greater than the input. All of the other cultures showed negligible increases in phage progeny produced compare to input phage. In 7-day old cultures, only those recovered in New media exhibited a high amount of phage progeny produced.

Fig. 1A shows the amount of progeny produced in cultures that were grown for 2 days and allowed to recover for 2h or 24h. Comparison of all of the cultures at both 2h and 24h showed a negligible difference in phage progeny produced compared to input phage. Fig. 1Bi and Bii show the amount of progeny produced in cultures that were grown for 4 days and allowed to recover for 2h or 24h. No data is available for the culture recovered for 2h in Old media. Cultures that recovered in Old + C media are the only cultures that exhibited a difference between the 2h recovery and the 24h recovery in the amount of phage progeny produced compared to input phage. The culture that recovered for 24h showed 3000 times more phage progeny produced compared to the culture that recovered for 2h. All of the other cultures did not show a marked difference in phage progeny produced whether they recovered for 2h or 24h. Fig. 1Ci shows the amount of progeny produced in cultures that were grown for 4 days and allowed to recover for 2h or 24h. Fig. 1Cii is a reproduction if this graph with a different scale. Cultures that recovered in New media are the only cultures that exhibited a difference between the 2h

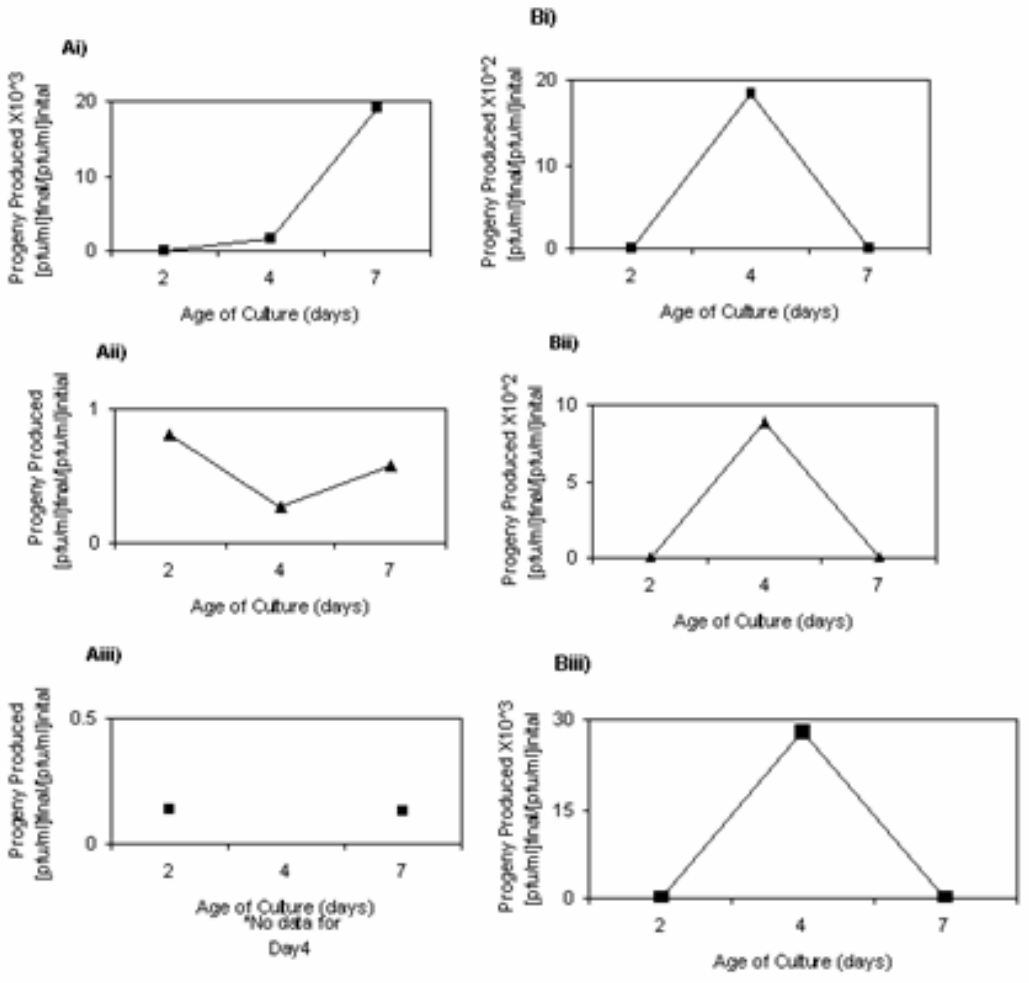

Fig 2: Affect of culture age on T4 Bacteriophage progeny production in *E. coli* ZK126 after (A) 2 hours of recovery or (B) 24 hours of recovery in (i) New media, (ii) Old + C media, or (iii) Old media. * In Figure 2 it is assumed that the days in between tested samples would follow the trends indicated.

recovery and the 24h recovery in the amount of phage progeny produced compared to input phage. The culture that recovered for 2h showed 31,000 times more phage progeny produced compared to the culture that recovered for 24h. All of the other cultures did not exhibit a substantial difference in phage progeny produced whether they recovered for 2h or 24h.

Fig. 2A shows the ability of T4 to infect *E. coli* cultures grown for 2, 4, or 7 days and recovered for 2h in various media. The 2-day old cultures produced the lowest amount of progeny (Fig. 2Ai). Since the difference between input and output phage in this sample is so little it most likely represents a negligible amount of phage produced. In the 4-day old culture, the progeny produced increased 1600 times more than in the 2-day old culture, and in the 7-day old culture the progeny produced increased by 19000 times more than the 2-day old culture. Fig. 2Aii shows the amount of T4 progeny produced in the cultures that recovered for 2h in Old + C media. The 4-day old culture had the lowest amount of progeny produced. The 7-day old culture had 2 times more progeny than the 4-day old culture. The Day 2 culture had approximately 2.5 times more progeny than the Day 4 culture. The difference in progeny production ratio between these samples is very low and is most likely negligible. Fig. 2Aiii shows the quantity of T4 progeny produced in cultures recovered in exhausted Old media for 2h. The Old media samples had a negligible change in progeny production between 2- and 7- day old cultures. There is no data for the 4-day old culture.

Fig. 2B shows the ability of T4 to infect *E. coli* cultures grown for 2, 4, or 7 days and recovered for 24h in various media. Fig 2Bi shows progeny produced from cultures recovered in New media. The 2- and 7-day old cultures had similar, negligible amounts of

progeny produced. The 4-day old culture had about 3500 times more progeny produced. Fig 2Bii shows the progeny produced from cultures recovered in Old + C media. It displays that the 7-day old culture produced the lowest amount of progeny. The 4-day old culture produced about 10 times more than the Day 2 culture. The 4-day old culture produced about 300 times more progeny than the Day 7 culture. Fig 2Biii shows the progeny produced from cultures recovered in Old media. It shows that 2- and 7-day old cultures produced the least amount of phage. The largest amount of progeny produced was in the 4-day old sample. It produced almost 30000 times more progeny than the 2- and 7-day cultures.

The positive control (exponentially growing *E. coli* ZK126) produced 21810 progeny. In comparison, the positive control produced more progeny than any experimental samples, except the 4-day old sample recovered in Old media for 24h.

DISCUSSION

It was hypothesized that cultures recovered in New media would have the highest recovery, followed by Old + C media, while cultures recovered in Old media were hypothesized to not recover. New media should have all of the nutrients needed for growth, with no waste products or other specific growth inhibitors released from stationary phase cells to inhibit growth. Cultures recovered in Old + C media should have the carbon necessary for cell growth as well as waste products that discourage growth. Cultures recovered in Old media should have metabolized all of the carbon present in the media and accumulated waste products that inhibit growth. As phage infect *E. coli* cells growing in exponential phase to a much greater degree than cells growing in stationary phase, we hypothesized that phage are better able to infect cells that are growing faster following recovery (1). Therefore, a positive control of cells growing in exponential phase would be easily infected by T4 and produce a high amount of phage progeny due to their rapid rate of growth. Due to the varying growth rates of the three cultures caused by the various media conditions we hypothesized that cells recovered in New media would produce the next highest amount of progeny, followed by cells recovered in Old + C media and then cells recovered in Old media. Furthermore, it has been suggested that stationary phase *E. coli* cells may release substances that inhibit infection by T4. These substances would only be present in cultures recovered in Old + C media and Old media.

All cultures grown for 2 days did not show a productive infection, so no comparisons regarding the effects of media type can be made. For 4- and 7- day old cultures recovered for 2h, only those cultures that recovered in New media exhibited high levels of progeny production. This is consistent with our hypothesis that infection inhibitors are present in Old + C media and Old media. With 4 days growth, all cultures that recovered for 24h showed a high increase in T4 progeny produced. This suggests that media components have little effect on the ability of T4 to infect cells. The culture that recovered in Old media showed the highest amount of progeny produced compared to the other media treatments, as well as the positive control culture. This can likely be explained by experimental error, as this sample may have been infected for longer than one hour, allowing for comparatively more burst events than in the other cultures. Overall, our data supports our hypothesis that New media promotes the highest level of progeny production. However, a repeat of this experiment will show whether our results are reproducible and if the deviations observed can be attributed to experimental error.

Two different hypotheses exist for the effect of culture age on the ability of T4 to infect *E. coli* ZK126. Firstly, it can be hypothesized that the older the cells, the less susceptible they would be to infection by T4. This could be in part due to an increased accumulation of waste products in the media and a reduction of available nutrients in cultures grown for longer periods of time. This would reduce the rate of cell growth and consequently reduce the ability of the cells to be infected by phage. Therefore, we would expect the progeny produced to be highest on Day 2 and decrease through Days 4 and 7. Alternatively, as demonstrated by Chan *et al.*, there may be a cyclic nature in the ability of T4 to infect *E. coli*. If this were the case, it was expected that the 2-day old cultures would display a resistance to productive infection, the 4-day old cultures would produce productive infections, and the 7-day old cultures would again lose susceptibility to productive infections.

After cultures were grown into stationary phase for 2 days, T4 was unable to produce a productive infection in any of the culture treatments. One possible explanation is that cultures grown for 2 days have not yet upregulated the genes necessary for high progeny production. Alternatively, T4 infection in these cultures may have been in a pseudolysogenic state. In a pseudolysogenic infection, phage are able to infect cells, but this infection does not result in a classic lytic cycle where phage multiply in a fraction of the bacterial population (1). The infection skips the lysis step and continues on within the same cell (3). In other words, cells under starvation conditions may enter a pseudolysogenic state whereby phage-infected cells lack do not undergo a lytic infection (8).

The 4-day old cultures could produce a productive infection in all media treatments (note there is no data

for the culture that recovered for 2h in Old media). The culture recovered in Old media for 24h produced more progeny than the positive control. This can has been explained before as likely due to experimental error. The culture recovered in Old + C media also produced a productive infection after a 24h recovery, although it produced only half as much progeny as the culture recovered in New media. This suggests that the media of cultures recovered in Old + C media contained inhibitory substances that inhibit a productive T4 infection. Overall, we hypothesize that cells grown for 4 days have a unique physiology and change their gene regulation, which results in the ability of phage to produce high levels of progeny.

The cultures that were grown for 7 days in stationary phase only produced a productive infection after a 2h recovery in New media. This suggests that after 7 days the cells are in a physiological state that does not support a productive T4 infection unless they have their media replaced. The productive infection of the New media treatment indicates that the cells grown for 7 days were not dead, and lack of progeny production in other cultures was due to a non-productive infection. Our data suggests that cultures grown for 7 days may be producing inhibitors that either inhibit infection by T4 or induce a pseudolysogenic infection that prevents a productive T4 phage infection.

We hypothesized that the differences in the rate of cell growth caused by either a 2h or 24h would be negligible. We thought that cell growth would be minimal in all cultures, and therefore the differences in a 2h or 24h recovery would not have a substantial impact on the ability of the cells to be infected by phage. We assumed that cell growth would be minimal in cultures recovered for 2h because 2 hours may not be long enough to observe a recovery. Stationary phase cells show adaptations to limiting environmental conditions, both by altering their protein synthesis and their physiological characteristics (5). It takes time for cultures to transition back into a state of exponential growth during recovery because cells must alter their protein synthesis and physiological characteristics. In addition, cells transferred to New media are shocked by the sudden increase in nutrients, which also delays their ability to return to exponential phase (14). For cultures recovered for 24h, we assumed that cell growth would be minimal because after 24hrs cells would again be in stationary phase (as defined by Chan *et al.* as > 18hrs). Alternatively, if cultures have not entered stationary phase by 24h, then we would expect these cultures to have a high rate of growth and consequently these cells should be easily infected by phage.

It was found by Chan *et al.* that 2-day old cultures could not support a T4 infection and did not produce progeny (3). However, the 4-5-day old cultures produced progeny equivalent to one lytic cycle (100 fold). The 6-8-day old cultures were not susceptible to T4 infection and did not produce progeny. Finally, the 9-day old cultures were pseudolysogenic (3). Our data, after a 24h recovery, supports the findings of Chan *et al*. According to Fig. 2B it seems that media type and recovery time do not have a great influence on the production of progeny when comparing our results those of unrecovered cells. The 4-day old cultures given a 24h recovery period in either New, Old + C, or Old media all produced higher levels of progeny than the other days, as the 2- and 7-day old cultures did not have a productive infection after a 24h recovery. As suggested by Chan *et al.*, cells grown into stationary phase for 4 days are physiologically unique and consequently allow phage progeny production. Since this data is identical to experiments conducted by Chan *et al.*, which did not include a recovery period, our results support the hypothesis that 24h of recovery does not affect the pattern of T4 infection over time.

Overall, we expected that cultures recovered for 2h or 24h cultures would show similar trends in the ability to be infected by phage. These trends would be dependent on other variables in our experiment, namely the type of recovery culture and the culture age. Fig. 1 shows that there are differences in progeny produced that are dependent on recovery time. However, as stated above, our results support the hypothesis that recovery does not affect the pattern of T4 infection over time. Therefore, our results appear to be contradictory, and we suggest that the effects of recovery time be explored further.

We have proposed several possible mechanisms to explain the changing susceptibilities of stationary phase *E. coli* to T4 productive infection that were witnessed in our experiments. Some of these mechanisms have been previously characterized and are described below. Hypervariability in stationary phase cells is a phenomenon that has been demonstrated in *E. coli* (14). It has been proven that there are genetic differences between differently aged stationary phase cells (14). An inhibitor that may be responsible for these observations is α-D-glucoside, which is a competitive inhibitor of glucose uptake (6). Accumulation of this prevents the use of glucose present in the media (6). Since the growth of viral progeny is directly related to bacterial growth, the accumulation of such compounds in the media can lead to decreased progeny production (6). The changing availability of carbon in the media can also account for the variability in productive T4 infection by affecting *E. coli* growth rate. In Hadas *et al.* it was observed that an increase in nutrients resulted in increased bacterial cell growth which therefore increased progeny production (6).

It should be noted that there are several anomalies in our observations. For example, no trend could be

drawn from the samples given a 2h recovery. The limitations of this experiment could have contributed to this. If cultures were in stationary phase after a 24h recovery, then all of the cultures would be producing the same inhibitory substances and therefore eliminating the effects of the various media recoveries. It should be noted that the experimental results in this study are based on a single replicate and are therefore not statistically relevant. For some samples, the amount of phage progeny produced was measured to be less than the amount of phage added to the samples, resulting in an output/input ratio of <1. This indicates experimental error, because even without a productive infection, the ratio should be 1. Also, the way that the cells were put into stationary phase was not representative of natural conditions, which makes the results not representative of the natural behavior of *E. coli*.

In summary, the current study indicates that (a) different ages of *E.coli* culture in stationary phase cause different effects in T4 progeny production, and (b) possible inhibitors are present in 7-day old cultures affecting the pattern of T4 bacteriophage infection.

FUTURE EXPERIMENTS

Further research into the ability of T4 bacteriophage to produce progeny in *E. coli* ZK126 of various culture ages and media types will add to the scope of this study. To determine more definitively the presence of inhibitors in Old media we suggest the following experiment: Grow stationary cultures for various lengths of time, remove the exhausted media, and grow fresh cells in this Old media. This will demonstrate the effect of media with inhibitors on young unstressed cells. As our data on the effects of recovery time was inconclusive, we recommend investigating the ability of phage to infect *E. coli* following various recovery times. We also suggest testing single variables per experiment. This will increase the ease of interpretation of the results.

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