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Escherichia coli Biofilm Development and Bacteriophage T4 Susceptibility

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Previous studies demonstrated that the varying physiological properties of the stationary phase cells of *Escherichia coli* can cause varying susceptibility to T4 at different ages of the culture. These studies did not take into account that stationary phase bacteria include both free-floating and biofilm cells. In this study, we sought to distinguish whether free-floating or biofilm cells were responsible for the unusual patterns observed. Glass tubes of LB medium inoculated with *E.coli* ZK126 were grown for 1 to 7 days. Free-floating cells were separated from the biofilm and both were infected with T4 Bacteriophage. A standard plaque assay was performed to determine the progeny produced upon infection of the cells. Infection of the free floating cells yielded low amounts of progeny when stationary phase was not yet established; once established, no viral progeny were observed. On the contrary, successful infection of the biofilm cells was observed on day 4 while very little progeny was observed on preceding and following days. This suggests that the peak of viral progeny observed in previous studies was due to changes of physiological properties of the biofilm rather than the free floating cells. The biofilm might have exhibited such a pattern due to OmpC over-expression at this stage of biofilm development. OmpC was previously proven to aid viral attachment to bacterial cells.

Studies in bacteriophage T4 infection of *Escherichia coli* are normally carried out during the log phase of growth and are generally well characterized. However, what is still not entirely known are the changes to the physiological properties of the cell and its ability to manufacture productive phage during the stationary phase, which more accurately represents the characteristics of these cells in nature (3).

During stationary phase, the first stage of *E. coli* biofilm adhesion appeared to be reversible since the bacteria can be removed from the surface by washing (12). In the early stationary phase, the production of extracellular polymers leads to the formation of a slimy layer on solid surfaces (8). This slimy layer known as a biofilm, was composed of microcolonies, cells surrounded by large amounts of exopolysaccharide (EPS) (7). In addition, research has revealed that bacteria are embedded in the polymer matrix and organized in mushroom-shaped microcolonies interspersed among less dense channels in which liquid flows (9). In their natural environment, many bacterial species live predominantly in these biofilm communities (7). Since microbial adhesion to solid surfaces is a very common phenomenon, biofilms develop on virtually every material that comes in contact with naturally occurring fluids, such as blood and seawater (13). In general, ability of T4 bacteriophage to produce progeny in stationary phase cells is limited when compared to exponential phase cells (3, 1). However, previous research (3, 1) suggested that as *E. coli* cells age in stationary phase, their susceptibility to T4 bacteriophage changed and increased numbers of infective centres were observed (3,1). Research by Chan *et al* (3) discovered that at days 1 and 2 of *E. coli* growth after stationary phase, phage replication appeared limited. Curiously, on days 4 to 5, *E. coli* was able to once again produce more phage. This change in susceptibility seemed to occur in a low-high-low-high cyclical fashion and the cause for this phenomenon remains unclear (3). In this experiment, we examined the effects and susceptibility of bacteriophage T4 on *E. coli* ZK126 biofilm and their ability to produce productive T4 bacteriophage. We compared the amount of infective centres produced between infection of biofilm and free-floating cells over a period of 7 days. In addition, we estimated the *E. coli* viability in the presence and absence of infection biofilm crystal violet stain of infected and non-infected biofilm (11).

MATERIALS AND METHODS

Bacteria Strain and Culture Maintenance. *Escherichia coli* strain ZK126 was supplied from the MICB 421 cluture collection in the UBC Department of Microbiology and Immunology (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*. All overnight cultures were prepared by inoculating 30 mL LB medium (10 g Bacto tryptone, 5 g Bacto yeast extract, and 10 g NaCl into 1 L of water) with a loopful of cells and incubated for 24 hours at 37°C in a shaking bath.

Preparation of T4 viral stock. Bacteriophage T4 was also obtained from the MICB 421 culture collection in the UBC Department of Microbiology and Immunology. T4 viral stock was

prepared using an overlay plating technique. Tubes containing 3 mL molten soft agar (12.93 g bacto tryptone, 8 g NaCl, 2 g sodium citrate, 1.31 g glucose, 7.5 g invitrogen agar into 1 L of water) were kept in a 50°C waterbath. Two hundred µL of an overnight *E. coli* ZK126 broth was added followed by 100 µL of a 10^6 particles/mL of bacteriophage T4 and phage mixed. The entire contents of the tube were then transferred to a plate containing 20 mL of T-phage nutrient agar (10 g bacto tryptone, 10 g invitrogen agar, 8 g NaCl, 1 g glucose into 1 L of water). The same procedure was repeated with 50 µL of 10^6 particles/mL, and 100 µL, 50 µL and 10 µL of the 10^5 particles/mL dilution. After 24 hours of incubation at 37°C, the overlays showing nearly constent plaque development were scrapped off and added to a 50ml centrifuge tube with 2-4 mL of LB medium. One to two hundreds µL of chloroform was then added. The overlays were soaked in this mixture for 72 hours. At that time, the contents were centrifuged (Beckman J2-21) at 11000g for 10 minutes to pellet the top agar. The supernatant with the phage was collected.

Plaque assay. The overlay plating technique was also used for the plaque assay. Two hundreds µL of overnight *E. coli* ZK126 was added to 3 mL molten soft agar. A volume of diluted phage suspension was pipetted into the molten soft agar to obtain final plated dilutions of 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}. The mixture was then poured onto a bottom agar plate(12.93 g Bacto tryptone, 8 g NaCl, 2 g sodium citrate, 1.31 g glucose, 15 g Bacto agar into 1 L of water). Plates were incubated at 37°C.

Biofilm quantification and viability count. Biofilm quantification was a modified version of the Pratt and Kolter biofilm assay (10). Each day, 6 test tubes were filled with 5 mL LB medium and inoculated with 50 µL of overnight *E. coli* ZK126. The tubes were then incubated at 37°C for 1-5 days without aeration. One mL of 1% crystal violet (CV) in water was added into the first 3 test tubes. After 15 minutes of incubation at room temperature, the tubes were washed thoroughly and repeatedly with distilled water, then 2 mL of 100% of ethanol solution was added to each tube to extract the CV from the stained biofilms. Then, the absorbance of each extract was measured at 570 nm with a Beckman Coulter DU 530 spectrophotometer. With the last 3 tubes, the media was removed, filtered by a syringe (Millipore MF-membrane filters, filter type 0.45µm HA, cat no. HAWP02500, lot no. R3JN25137). The remove suspended bacteria put back into the rinsed test tubes. The tubes were then vortexed at maximum speed for 3 minutes to re-suspend the biofilm into the old filtered media. Finally, bottom agar plates were spread with 100 µL and 10 µL of 10^{-6} diluted culture and 100 µL of 10^{-4} diluted cultures from each last 3 tubes. The plates were incubated for 24 hours at 37°C.

Preparation of biofilm for the infection. Each day, 10 test tubes were filled with 5 mL LB medium and inoculated with 50 µL of overnight *E. coli* ZK126. The tubes were then incubated at 37°C for 1-7 days.

CV staining for estimating the colony size. For each day, 1 tube was stained with 1% CV. After 15 minutes of incubation at room temperature, the tubes were washed thoroughly and repeatedly with distilled water. Two mL of 100% of ethanol solution was then added to the tubes, and the absorbance in the ethanol extract was measured at 570 nm.

Infection of the *E. coli* biofilms. For each day, 2 tubes were used to examine the infectability of the biofilm and 4 tubes were used to prepare clarified old media. The media were removed, filtered with a syringe and transferred into new test tubes.The first two culture tubes were rinsed with distilled water. Then 5 mL of old filtered medium was added back into each rinsed culture tubes. The tubes were than infected with bacteriophage from the T4 viral stock at a final concentration of 10^4 PFU/mL. The tubes were incubated for 5 minutes at room temperature. One mL of the 2 solutions were transferred into 1.5 mL microfuge tubes and centrifuged at 7000g for 2 minutes. The supernatant was removed and 1 mL of old media was utilized for resuspending the pellet. The pellet was washed for two more times. The final resuspended pellets were transferred into glass tubes and then were incubated without aeration for 60 minutes at 37°C. Plaque assays were performed with a final plated dilution of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} for the 2 samples. The plates were incubated for 24 hours at 37°C.

Chloroform test. Before the samples were incubated for 60 minutes, 300 µL of the resuspended solution was taken from one of the two samples, mixed with 5 µL of chloroform and incubated for 10 minutes. Plaque assays were performed with a final plated dilution of 10^{-1} and10^{-3} and incubated at 37°C for 24 hourss to dertermine the number of unattached, chloroform resistant viruses in the sample. The concentration obtained was used as a control and substracted from the total PFU/mL at the end.

Infection of the free-floating *E. coli*. For each day, 2 tubes were used to test the infectability of the suspended *E. coli*. The medium from each sample were transferred into new test tubes. The tubes were than infected with bacteriophage from the T4 viral stock at a final concentration of 10^4 PFU/mL. The tubes were incubated for 5 minutes at room temperature, then 1 mL of the 2 solutions were transferred into 1.5 mL microfuge tubes and centrifuged at 7000g for 2 minutes to remove the unattached T4. The supernatant was then removed and 1 mL of filtered old media was utilized for resuspending the pellet. The pellet was washed for two more times afterwards. The resuspended solutions were transferred into glass tubes and were incubated for 60 minutes at 37°C. Plaque assays were performed with a final plated dilution of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} for the 2 samples. The plates were incubated for 24 hours at 37°C.

CV staining of infected biofilm. For each day, 1 tube was used for the CV staining of infected biofilm. The medium was removed, filtered with a syringe and transferred into new test tubes. Then 5 mL of old filtered medium was added back to each of the original tubes. The tubes were than infected with bacteriophage from the T4 viral stock at a final concentration of 10^4 PFU/mL, and were than incubated for 60 minutes at 37°C. The tubes were stained with 1% CV. After 15 minutes of incubation at room temperature, the tubes were rinsed thoroughly and repeatedly with distilled water, then 2 mL of 100% of ethanol solution was added to the tubes to extract the bound CV, and the absorbance in the extract was measured at 570 nm.

RESULTS

The ability of T4 Bacteriophage to infect free floating ZK126 *Escherichia coli* in stationary phase decreases as age of the culture increases. In order to study the significance of T4 bacteriophage infectivity in biofilms, we first examined the change in the ability of T4 to infect free-floating bacteria in stationary phase. The *E.coli* was grown for 1 to 7 days and the ability of T4 Bacteriophage to produce viable progeny was monitored. Figure 1 shows the growth of the *E.coli* ZK126 over the 7 day period. Culture concentration increased for the first two days but gradually reached a plateau starting around the third day. This indicates that stationary phase was established on around day 3. Chan *et al* (3) showed that 1 OD_{600} = 5.41 x 10^8 cells/ml. Since the curve levelled at around 1.1 OD_{600}, we estimated that the concentration of the *E.coli* ZK126 is around 5.95 x 10^8 cells/ml at stationary phase. Figure 2 shows the effect of culture age on the number of viable progeny that was produced upon the infection of *E.coli* ZK126 for one hour by 10^4 T4 bacteriophage. The concentration of observed phage was highest on day 1 at approximately 4 x 10^4 pfu/ml. The number decreased almost 50% on the second day and decreased ten folds on the third day and reached nearly negligible amounts

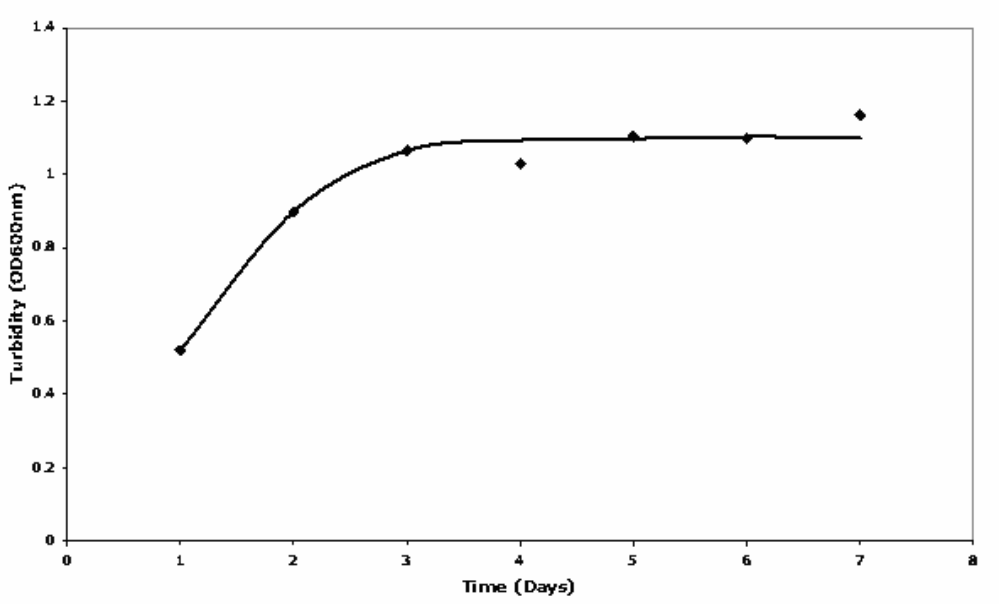

FIG. 1 Growth of ZK126 *E.coli* ZK126 in LB medium at 37°C.

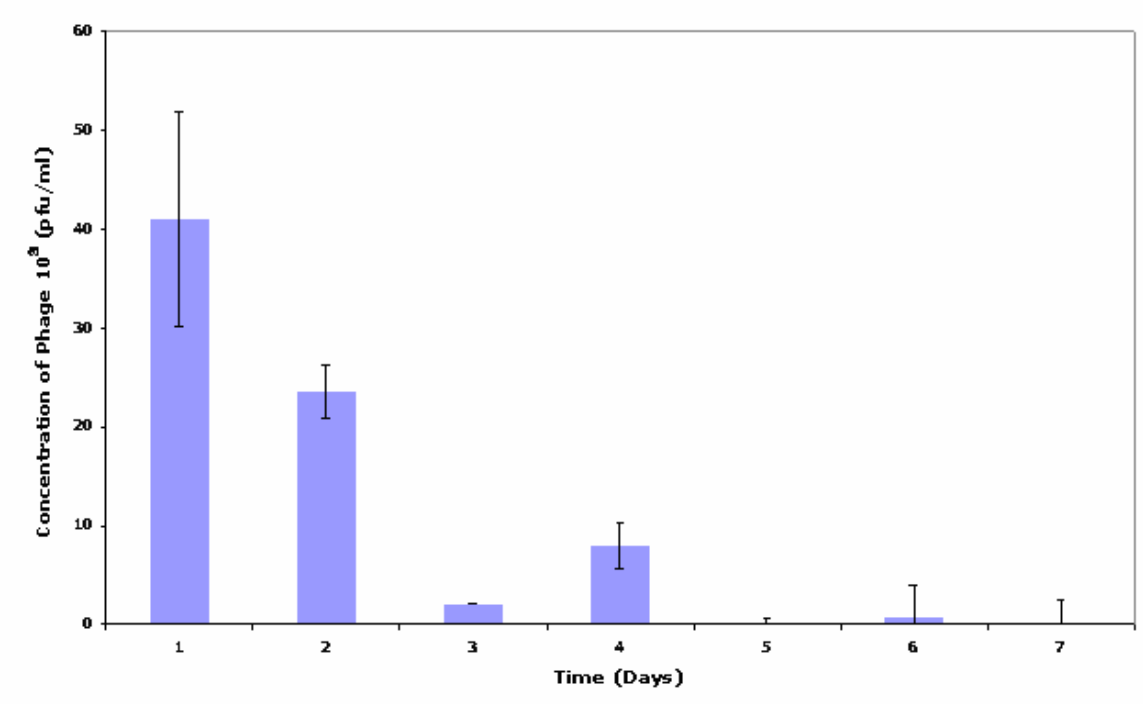

FIG. 2 Concentration of bacteriophage progeny obtained after infection of 1 to 7 day old free-floating ZK126 E.coli with T4 bacteriophage.

on day five, six and seven. There was a significant number of progeny at approximately 8 x 10^4 pfu/ml on day 4. The number of viable progeny increased 4 fold relative to the progeny number for day 3. In making figure 2, duplicates of four dilutions were done for the plaque assay of two different samples. The data collected to produce figure 2 showed a rather high degree of variation (standard deviation of the data was high). Although variation was high, results still generally showed the pattern seen in figure 2.

The number of viable cells in *E. coli* ZK126 biofilm decreased upon infection with T4

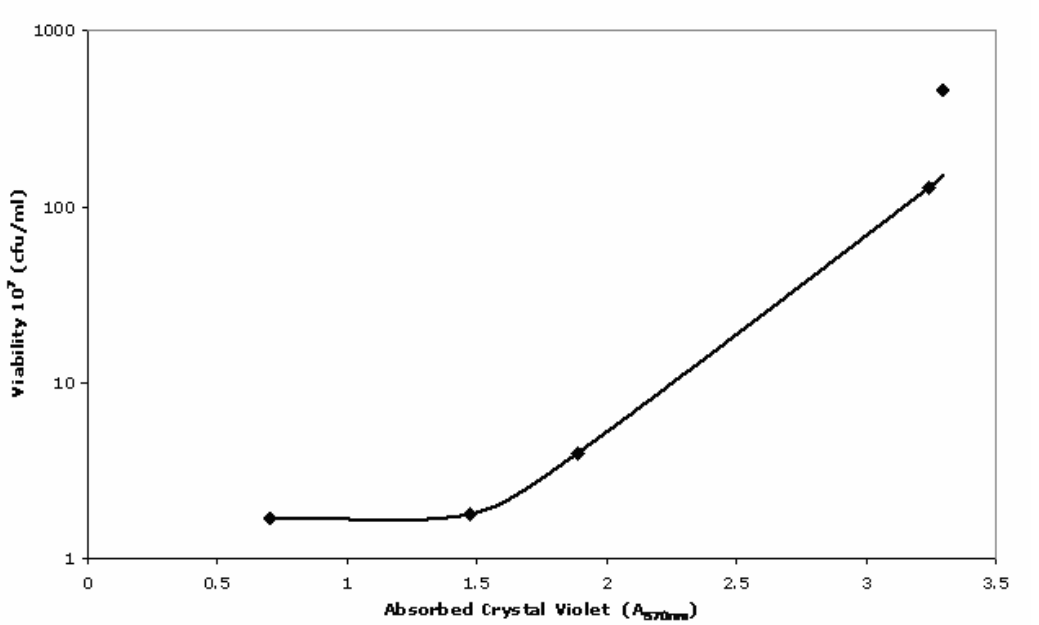

FIG. 3 Concentration of viable cells in ZK126 E.coli biofilms in relationship to absorbance readings of the biofilms stained with 1% crystal violet for 15 minutes.

bacteriophage. Figure 3 correlates the concentration of viable cells in a biofilm to the concentration of absorbed crystal violet. This graph allowed the absorbed crystal violet be used to estimate the concentration of cells available for infection in the biofilm and the concentration of cells that remained after infection by T4 bacteriophage (TABLE 1; FIG.4). Since the accuracy of the spectrophotometer decreases at higher readings, the reliability of the curve is questionable near the upper end of the curve. The relationship between viable cells and absorbance shows a levelled trend at low absorbances (FIG.3). At around 1.4 A_{570}, the pattern starts to ascend linearly. The linear line extends to about 3.2 A_{570}. The trend suggests that there is no useful correlation between binding of crystal violet and cell concentration for low concentration biofilms. At higher values, the bound crystal violet might have increased as viable cell count increases.

Looking at figure 4, it can be seen that both pre- and post-infection cells showed a similar trend on the first three days. On this part of the graph, post-infection cell concentration consistently dropped by approximately 30% compared to the un-infection readings. After day 3, the two trends initially started to increase uniformly. On day 4, the two trends started to diverge, where the bound crystal violet by un-infected biofilm continued to increase but the binding of crystal violet by post-infection formed a plateau at the end. It can also be seen that the value for post-infection day 7 is much lower than the value for day 6. This sudden drop is not what we expected and could be due to an error that may have occurred. Only one set of samples were measured and no duplicates were performed. Hence, the values may not be very accurate.

Looking at Table 1, we observed that the cell count of the un-infected biofilm maintained at the same level for the first three days. Once we entered day 4, biofilm population started to increase in number quite rapidly. From day 3 to day 4, concentration of viable cells increased 17%. From day 4 to day 5, concentration of viable cells nearly increased by a 100%. From day 5 to 6, an increase of 43% was observed; and a tremendous jump of nearly 200% from day 6 to 7. Therefore, the number of viable cells available for infection maintained a stable number up to day 3, than increased drastically from the day 4 point. The phage-infected biofilm exhibited no change for the first three days as well. We also noticed the number of viable cells is the same before and after infection by T4 Bacteriophage. However on day 4, concentration dropped by approximately 14% after infection. On day 5, the number of viable cells dropped 50% from its initial amount before infection. On day 6, an even larger drop of 65% followed by an 88% drop on day 7. These numbers indicates that upon infection, there was no lysing or unnoticeable lysing of cells for the first 3 days. Once the biofilm started to grow on day 4, killing of cells was observed. It can be seen that the rate in which the biofilm grew was proportional to the number of cells that were lysed.

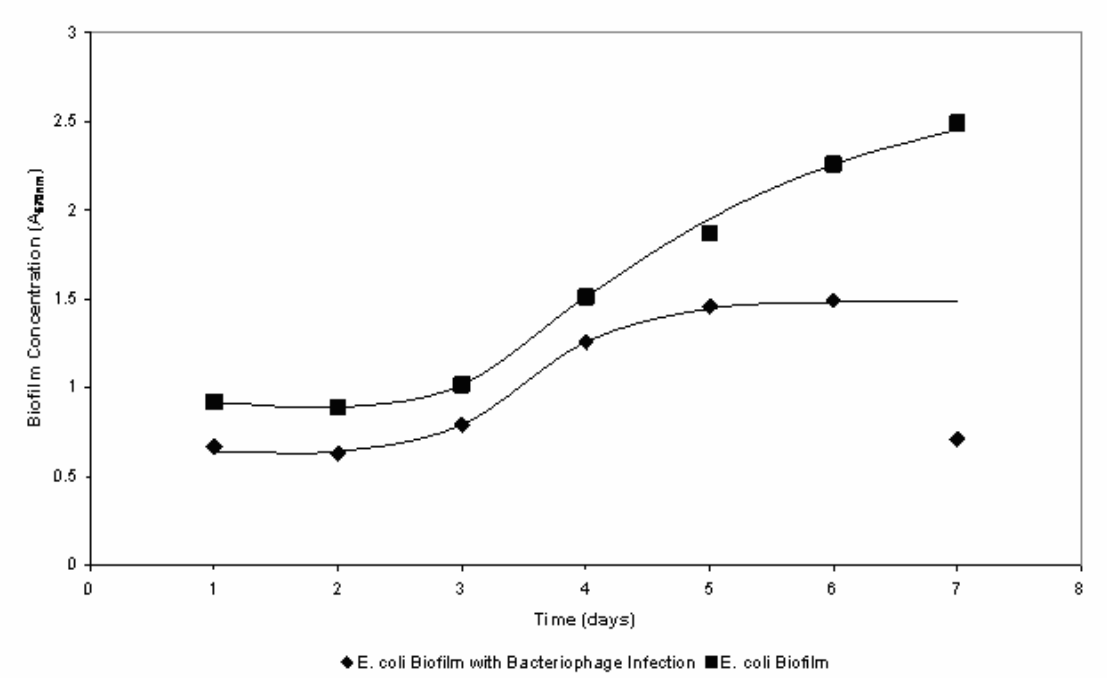

FIG. 4 Effect of 60 minutes of infection with T4 bacteriophage on the concentration of cells in ZK126 E.coli biofilms aged 1-to-7 days. Cells were stained with 1% crystal violet for 15 minutes.

TABLE 1. The concentration of viable cells in ZK126 E.coli biofilms aged 1 to 7 days that were treated for one hour with T4 bacteriophage.

Day	Viable Non-Infected *E.coli* biofilm cells (10^7 cfu/ml)	Viable *E.coli* in infected biofilm (10^7 cfu/ml)	Change in viability (10^7 cfu/ml)	% change in viability
1	3	3	0	0
2	3	3	0	0
3	3	3	0	0
4	3.5	3	0.5	17
5	6.9	3.4	3.5	100
6	9.9	3.5	6.4	182
7	28	3.5	24.5	700

T4 Bacteriophage has the ability to infect cells of *E. coli* ZK126 Biofilm and produce viable progeny. Figure 5 shows the number of progeny T4 that is produced after infection of the biofilm. We observed a moderate level of progeny on day 1 at around 3 x 10^4 pfu/ml. The number of progeny dropped by approximately 50% on day 2 relative to day 1 and dropped by approximately 80% on day 3 relative to day 2. This means that although the cells were supposed to be in stationary phase, a productive infection was still established. Up to this point, the ability to produce progeny seemed to decrease as age of the biofilm increase. On day 4, there was a drastic increase in progeny numbers to a high peak at around 7.5 x 10^4 pfu/ml. This was what we were expecting, because according to Table 1, the biofilm starts to grow on this particular day and lysing of cells also began on day 4. Studies done by Chan *et al* (3) also demonstrated that infection of stationary cells was highest on day 4 and 5 old cells. Although table 1 indicates that a significant portion of the biofilm was lysed, there was not a significant number of progeny observed on the following days. Around 2 x 10^4 pfu/ml of progeny was observed on day 5, a more noticeable amount of approximately 8 x 10^4 pfu/ml on day 6 and a negligible amount on day 7. This disagrees with what we saw in Table 1 because more cells were lysed in comparison to day 4. Thus we anticipated seeing even more progeny being produced on day 5, 6 and 7. However, Chan *et al* (3) also saw a similar drop in progeny number in their studies on T4 infectivity on stationary phase cells after day 4 and 5. Thus, we did expect to see a drop in number. The same plaque assay procedures were used to measure progeny number for both free floating and biofilm infection samples. Again, a high amount of variation was observed. However, similar to the free floating assay, the results generally demonstrated the same pattern seen in figure 5 even though a high degree

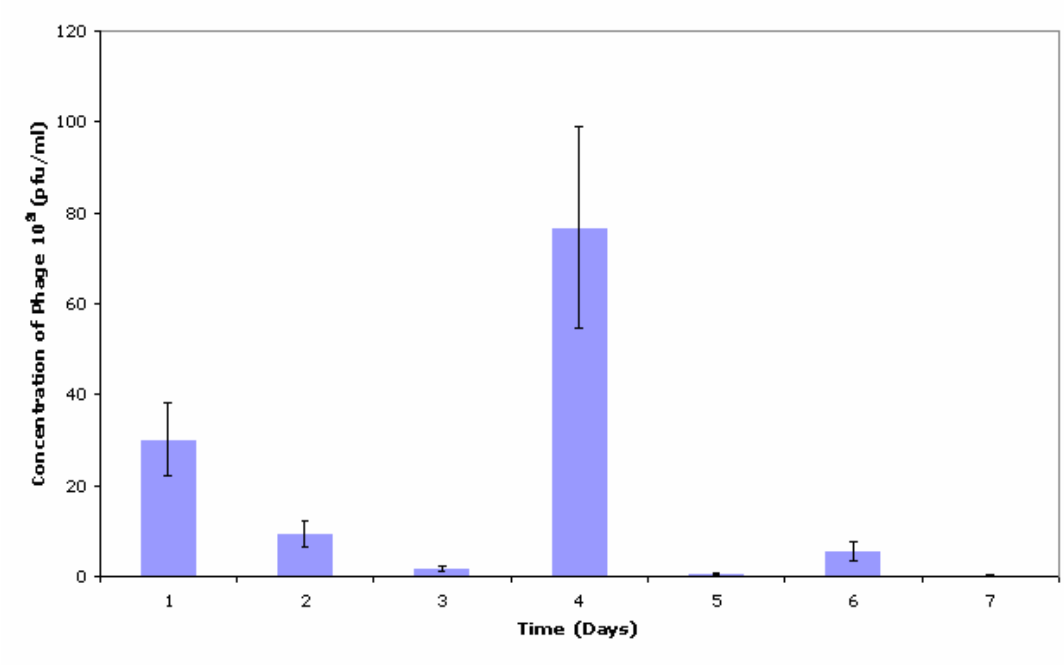

FIG. 5 Concentration of bacteriophage progeny obtained after 60 minutes of infection of 1-to-7 day old ZK126 E.coli biofilm with T4 Bacteriophage.

of variation was observed. A chloroform test was performed for this part of the experiment and was used as a control to ensure that the observations were due to bound bacteriophage rather than unbound bacteriophage that carried over from the T4 added to initiate the infection.

DISCUSSION

As stated by Chan *et al* and Braun *et al* (1,3), it was suggested that *E. coli* susceptibility to T4 bacteriophage changed depending on the age they were infected. However, the experiments by Chan *et al* and Braun *et al* used whole cultures and their results did not distinguish between free-floating and biofilm cells (1,3). In our experiment, we found that the change in susceptibility was not from the result of free floating bacteria. As we can see in figure 2, the concentration of bacteriophage recovered when aging *E. coli* ZK126 were infected on different days decreased exponentially over the period of 7 days. However, the period of 1 to 3 days old *E. coli* ZK126 does not represent the stationary phase. Indeed, the turbidity (FIG. 1) shows that the bacteria were slowly growing. This slow growth may have been due to the lack of aeration of the test tube during incubation. Therefore, as we can see from figure 1, it takes an additional 3 days before the bacteria can reach stationary phase. This would explain the progressive decrease in bacteriophage susceptibility we see in figure 2. As the *E. coli* growth slowed to stationary phase, the concentration of phage progeny recovered decreased as well. During the period of 3 to 7 days, there was no significant amount of bacteriophage recovered from *E. coli* ZK126. The data suggests the concentration of phage recovered is inversely proportional to the rate of growth of *E. coli* ZK126. However, in figure 2, there were 2 small peaks at days 4 and 6. We do not believe these results were from the free floating bacteria and will be further discussed below. We conclude that free-floating cells at stationary phase are not susceptible to T4 bacteriophage.

Our findings on biofilm growth (FIG. 5) confirm the variation in T4 bacteriophage susceptibility of *E. coli* ZK126. Again, days 1 to 3 does not represent the stationary phase of biofilm growth. As previously mentioned (FIG. 1), our samples of *E. coli* ZK126 appear to be still slowly growing in a diminishing manner, explaining the decrease of susceptibility. This suggests the washing may have been insufficient and as a result, some free-floating cells remained in the sample allowing susceptibility data to appear similar the free-floating phase of growth. This in turn reflects the diminishing phage count from days 1 to 3. Furthermore in figure 4 and table 1, the absorbance and estimated concentration of biofilm growth does not appear to increase in the first 3 days. This confirms previous studies that biofilm growth only occurs during stationary phase (7,10). Only after day 3 (FIG. 4) appears the onset of biofilm formation, which can be

correlated with an increase in *E. coli* ZK126 viability (Table 1). Interestingly a significant amount of T4 bacteriophage were recovered at days 4 and 6 which supports the findings increased bacteriophage susceptibility by Chan *et al* (3) and Braun *et al* (1). Previous research (6,11) have suggested that biofilm protein expression differs considerably compared to free-floating cells at stationary phase. In addition, protein expression is not believed to be consistent throughout the biofilm stage of growth (11). The physiological differences appear to vary the greatest at early stages of biofilm growth (6), which we hypothesize to increase the ability of bacteriophage infection (FIG. 5). *Pseudomonas aeruginosa*, another biofilm producer was found to have 5 distinct stages of biofilm growth with specific changes in protein expression (12): i) reversible attachment, ii) irreversible attachment, iii) maturation -1, iv) maturation -2, v) dispersion. Furthermore, the study found that there is a profound difference of protein pattern compared to free-floating cells at maturation 1 and 2 (12). Finally at the dispersion stage, the protein pattern is more similar to free-floating cells (12). This may suggest that at day 4, *E. coli* ZK126 was at the maturation stage of biofilm growth. Indeed, the point of time *E. coli* ZK126 reaches the maturation period depends on the amount of time required to reach stationary phase. Schembri *et al* (11) has found a list of genes significantly altered in expression during biofilm growth. In particular, OmpC was over-expressed (11). OmpC is an outer membrane protein that is involved in regulating the response of *E. coli* to the osmolarity of its environment (2). In addition, OmpC plays an essential role in viral processes that follow binding (4) and infectivity was found to be poor in strains of *E. coli* lacking this protein (14). An over-expression of OmpC at day 4 may therefore increase the susceptibility of T4 bacteriophage attachment and infection. As a result, there will be an increase in T4 bacteriophage recovered. However, as culture age increases, the *E. coli* will reach the dispersion stage, mirroring the protein pattern of the free-floating stationary phase, thus limiting the amount of susceptible cells.

Even though the biofilm protein expression is more similar to free-floating cells at stationary phase after day 4, we still see in an increase in phage recovery on day 6 (FIG.5). This insignificant increase is most likely due to environmental variables or possible errors. In addition, from days 4 to 7 (FIG. 4, TABLE 1) the differences in *E. coli* ZK126 viability increases significantly between pre-infected and post-infected cells. However, the change in viability cannot account for the amount of bacteriophage recovered (FIG. 5). This may be due to the inaccuracy in correlating *E. coli* ZK126 viability and crystal violet absorbance from the biofilm-staining assay (FIG. 3). Indeed, there were not enough data to generate a meaningful graph and there is a sizable gap between absorbance readings from 2 to 3 OD_{570}. As a result, there may have been variations in the curve and not simply a straight line.

Bacterial biofilms have been found in hospitals, which can form on catheters, prostheses, and in so doing so; potentially cause persistent, antibiotic-resistant infections (5). Treatment of these biofilms has continued to be problematic with no clear solution in the future (6). To this day, it has not been possible to design a nontoxic coating method to prevent biofilm formation (13). Knowing this, it may be possible to attack the biofilm with a combination of bacteriophage species at an early stage and prevent buildup, thereby possibly preventing many nosocomial infections.

FUTURE EXPERIMENTS

Future experiments may include further infections between days 3 to 4 and 4 to 5 may help to better illustrate the effects biofilm growth and infectability. Furthermore, the correlation of *E. coli* ZK126 and the crystal violet absorbance assay should be further refined for a more accurate representation of biofilm viability. Finally, OmpC protein concentrations should be tested against *E. coli* ZK126 culture age and correlated with susceptibility of T4 bacteriophage infection.

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