Journal of Experimental Microbiology and Immunology (JEMI) Vol. 12:72-78

Comparison of T4 Bacteriophage Adsorption and Progeny Production during Infection of Maturing Biofilms of *Escherichia coli* Wild-type and OmpC Mutants

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Previous research into T4 bacteriophage infection of *E. coli* biofilms observed that during the fourth day of biofilm development, a transient substantial increase in T4 phage progeny production occurs. This increase was hypothesized to be due to over-expression of the outer membrane protein C (OmpC). OmpC is used by T4 bacteriophage for binding to the host cell, therefore its over-expression in biofilm cells could lead to increased adsorption by T4 bacteriophage. This study investigated whether the adsorption of T4 bacteriophage to *E. coli* biofilms of various ages could be correlated to the production of phage progeny from infected biofilms. *E. coli* C149 and C157 cells, wild-type and OmpC mutant respectively, were grown into biofilms of various ages and subsequently inoculated with T4 bacteriophage to measure T4 adsorption to the biofilms and T4 progeny production. Although T4 adsorption was found to be highest for the four day-old biofilms, no correlation was observed between the amount of T4 adsorbed and the amount of phage progeny produced. Adsorption of T4 bacteriophage to *E. coli* biofilms was not observed to be OmpC-dependent and was found to decrease with biofilm age. Further, it appears that *E. coli* C157 biofilm cells are more capable of supporting phage progeny production than *E. coli* C149 biofilm cells.

T4 bacteriophage lytic infection of *Escherichia coli* is well categorized during exponential growth, but has not been extensively studied in biofilms. T4 infection changes as cells enter stationary phase (7), but in biofilms, this is further complicated by characteristics such as extracellular protein matrices (6). As biofilms mature, differentiation occurs in the microcolony clusters, forming different 3-dimentional structures (8). Chan *et al.* showed that T4 infection of *E. coli* ZK126 biofilms were characterized by a substantial increase in phage progeny production on the fourth day of biofilm growth (6). However, this pattern was not seen in the free-floating stationary phase cells (6). This pattern of T4 infection in biofilms warrants further research.

Viral lytic infection generally involves five main stages: binding, replication, translation, assembly, and budding (2). Each of these stages is critical for T4 phage progeny release. Therefore the patterns seen by Chan *et al.* could have been caused by a disruption at any of these critical points (6). In addition to the differentiation of microcolonies, *E. coli* gene expression is altered as the biofilms mature (11). In particular, OmpC, an outer membrane protein involved in osmolarity regulation in *E. coli*, is over-expressed during biofilm growth (11).

In this experiment, we were interested in the T4 bacteriophage attachment pattern to wild-type *E. coli* biofilms that were 3 to 6 days old. We hypothesized that the observed increase in viral progeny production seen in 4 day-old *E. coli* biofilms (6) was due to an increase in T4 attachment. By measuring initial phage adsorption to biofilms composed of *E. coli* C149 cells and measuring the progeny produced 60 minutes post-infection, we hoped to correlate the pattern of phage adsorption to the pattern of phage progeny production. A positive correlation would provide support for our hypothesis. Furthermore, an OmpC knockout mutant was tested to see if the protein had an effect on virus progeny production on 4 day-old biofilms because it has been observed that T4 bacteriophage could not infect *E. coli* OmpC mutants growing in exponential phase (13).

MATERIALS AND METHODS

Bacterial strains and cultures. Table I summarizes the bacterial strains used. All strains were obtained from The UBC Department of Microbiology and Immunology MICB 421 Culture Collection (Vancouver, BC, Canada). *E. coli* C149 and *E. coli* C157 were grown as biofilms; *E. coli* B23 was used as an infection host for plaque assays of T4 phage adsorption and progeny production experiments. Supplied cultures were passaged twice and maintained on Luria Burtani agar plates (LB) (10 g/L Bacto tryptone, 5 g/L Bacto yeast extract, 10 g/L sodium chloride, 15 g/L Invitrogen Agar for plates, pH 7.2). Newly streaked plates were incubated overnight at 37°C and maintenance plates were sealed with parafilm and stored at 4°C. Overnight cultures were used for both biofilms and phage plating; a single colony was transferred

Name	Strain	Phenotype	Genotype	Use
Escherichia coli	**B23**	Wild-type	N/A	Infection host for plaque assays.
Escherichia coli	**C149**	Wild-type	SexF-, proC-24, aroA-357, his-53, purE-41, ilv-277, met-65, lacy-29, xyl-14, rpsL-97, cycA-1, cycB-2?, tsx-63, lambda-	Biofilms
Escherichia coli	**C157**	OmpC Mutant (protein 1b)	SexF-, proC-24, aroA-357, his-53, ompC-264, purE-41, ilv-277, met-65, lacy-29, xyl-14, rpsL-97, cycA-1, cycB-2?, tsx-63, lambda-	Biofilms

Table I. Summary of bacterial strain characteristics and experimental applications.

from a maintenance plate into 10 ml of LB broth in a 18x150 mm sterile glass tube. Overnight cultures were grown at 37˚C overnight on a tube roller at 23 rpm (7cm radius).

Overlay plaque assays. Phage were diluted in Tryptone Sodium Glucose broth (TSG broth: 0.5 g/L Bacto tryptone, 3.0 g/L sodium chloride, 7.0 g/L Bacto yeast extract, and 1.3 g/L glucose). TSG bottom agar plates (prepared by The UBC Department of Microbiology and Immunology Technical Support: 13.0 g/L Bacto tryptone, 8.0 g/L sodium chloride, 2.0 g/L sodium citrate, 1.3 g/L glucose, and 15.0 g/L Invitrogen agar) were warmed at 37˚C for 30 minutes prior to use. TSG top agar tube aliquots (prepared by The UBC Department of Microbiology and Immunology Technical Support: 13.0 g/L Bacto tryptone, 8.0 g/L sodium chloride, 2.0 g/L sodium citrate, 3.0 g/L glucose, and 7.5 g/L Invitrogen agar, dispensed as 3 ml to 13x100 mm glass tubes) were melted at 100ºC in boiling water and maintained in a molten state at 48ºC using a waterbath (Lab-Line Instruments, Cat. No. 3000-2). Before pouring each plate, 100 µl of both *E. coli* B23 overnight culture and diluted phage were added to the TSG molten top agar. These tubes were mixed by phage-style mixing, poured immediately onto warmed TSG bottom agar plates, cooled for 30 minutes at room temperature, and incubated at 30˚C for 18 to 24 hours. Each dilution was plated in duplicate. Plaques on plates with 30 to 300 pfu were counted using a colony counter (New Brunswick Scientific).

T4 viral stock. Initial T4 *E. coli b*acteriophage stock was supplied by The UBC Department of Microbiology and Immunology MICB 421 Culture Collection (Vancouver, BC Canada). The working stock was prepared from overlay plates of 10^0, 10^{-2}, 10^{-4}, 10^{-6}, and10^{-8} serial phage dilutions, as described for overlay plaque assays. Top agar from plates with confluent plaque formation was scraped away with 1.0 ml of TSG broth and collected in a sterile 50 ml centrifuge tube. Scraped plates were rinsed with an additional 1.0 ml of TSG broth which was added to the collected top agar. The centrifuge bottle was mixed occasionally for 30 minutes after the addition of 100µl chloroform, incubated at 4˚C for 72 hours, and then centrifuged at 11,000 g for 10 minutes (Beckman J2-21 Centrifuge, JA-20 rotor). Supernatant was transferred to a sterile 13x100 mm glass tube and treated with 200 µl of chloroform. This stock was titred by overlay plaque assay at 7.7 x 10^9 pfu/ml. Phage were stored at 4ºC.

Biofilm preparation. Overnight cultures of *E. coli* C149 and C157 were each diluted with LB broth to a standard turbidity of 1.0 OD_{600nm} units (Spectronic Instruments Spectronic 20D+ spectrophotometer). Sterile 13x100 mm glass tubes were inoculated with 3.0 ml of diluted culture and incubated in a 37ºC covered water bath (Fisher Scientific Isotemp 210) without aeration for 3, 4, 5, or 6 days. Four tubes of each biofilm type (strain and age) were established for a full experiment: T4 infection (1 tube), clarified media (2 tubes), and enumeration (1 tube). Preparation of biofilms was staggered so that all biofilm ages were assayed in a single day.

Enumeration of biofilms. The media was decanted from tubes with biofilms. The residual biofilm was then washed 3 times with 3.0 ml sterile saline (9g/L NaCl), and resuspended in 3.0 ml sterile saline by vortexing for 2 minutes (VortexGenie 2). Cell suspensions were serial diluted 10^{-2}, 10^{-3}, 10^{-4} in sterile saline and 100 µl of each dilution were spread-plated in duplicate on LB agar plates. Plates were incubated at 37˚C for 18-24 hours and those with 30 to 300 colonies were counted. Standard Gram-staining and colony morphologies identified colonies as *E. coli.*

T4 infection of biofilms. Biofilms were infected with T4 and free phage were assayed in the adsorption standard, phage adsorption, and phage progeny experiments. Media from 2 biofilms (6ml) of each type (strain and age) were centrifuged separately for 5 minutes at 1700 g (IECCentra-4B Centrifuge, IEC 8/86 Cat. No. 819 rotor) in 10 ml centrifuge tubes (Nalgene Oak Ridge) to pellet free-floating cells. Resulting supernatants were filtered through sterile 0.45 µm Millipore HA filter-mounted 30ml syringes into sterile 13x100 mm glass tubes. Separate biofilms of each type were decanted free of media, rinsed 3 times with 3 ml sterile saline, and then 5 ml of the corresponding clarified media was added to each tube. These tubes with the assay biofilms were inoculated with 5 x 10^4 pfu/ml T4 phage and incubated at room temperature on a tube roller at 23 rpm (7 cm radius). Free-floating phage were sampled at times appropriate for each assay, immediately placed on ice to cool, then centrifuged at 2300 g for 5 minutes at 4ºC (Eppendorf 5414D Centrifuge). Supernatants were collected, serially diluted with ice-cold TSG broth, and enumerated in duplicate by overlay plaque assays for pfu.

T4 adsorption time course. Four day-old biofilms were infected and sampled as described above. Samples of 200 µl were removed every 3 minutes for 15 minutes, starting at the time of inoculation (T = 0 min) and sample supernatants were serially diluted 10^{-1} to 10^{-4} before overlay plating in duplicate for plaque assays.

T4 adsorption at 9 minutes and progeny production at 60 minutes post infection. Three, four, five, and six day-old biofilms were each infected and sampled as described above. Samples of 200 µl from each biofilm assay tube were removed at both 9 minutes and 60 minutes post-infection. Sample supernatants were serially diluted 10^{-1} to 10^{-4} (9 min samples) or 10^0 to 10^{-2} (60 min samples) and plated in duplicate by overlay plating for plaque assays.

RESULTS

Maximum measurable T4 adsorption to four day-old *E. coli* C149 biofilms occurs at 5 minutes post-inoculation. To assess the relative adsorption of T4 phage to different biofilms, measurable adsorption was first optimized by creation of an adsorption time-course. A crude initial 12 minute time-course of T4 adsorption to a four day-old wild-type biofilm showed a decline in free-floating phage over the first 9 minutes of infection. Based on this data, subsequent adsorption experiments sampled adsorption levels at 9 minutes post-infection. A statistically valid 15 minute second time-course showed a similar decline in free-floating phage over the first six minutes of infection, however phage levels had increased slightly by 9 minutes. Figure 1 represents the average of these two experiments, assuming 10% error for the first assay. An initial decline of 1.6 x 10^4 pfu/ml (30%) occurred in the first 5 minutes of infection, followed by a gradual, non-linear increase in free-floating phage to 15 minutes. By 9 minutes post-infection, free-floating phage levels had returned to 81% those at the time of inoculation.

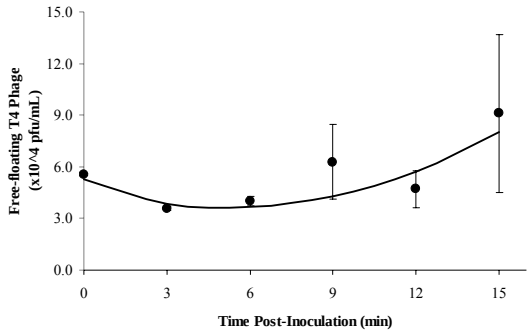

FIG. 1. Time course of T4 phage adsorption to C149 wild-type *E. coli* four day-old biofilms at room temperature. Biofilms were inoculated with 5 x 10^4 pfu/ml in clarified biofilm supernatant on a tube roller at 23 rpm. Error bars represent the mean absolute deviation of plaque assay replicates measured in two experiments.

Viable cell counts do not increase in *E. coli* biofilms with biofilm age. Biofilms were enumerated to assess biofilm growth and maturation. Figure 2 diagrams the viable *E. coli* cell composition of biofilms following 3, 4, 5, and 6 days of incubation at 37ºC. Viable cell counts represent adherent cells that were resuspended in sterile saline and grown on LB agar plates at 37ºC for 18 to 24 hours. No net difference of *E. coli* C149 wild-type cell concentration was counted across days 3 to 5, but a 44% decrease in viable cell concentration in the biofilm was measured between the 5 and 6 day-old biofilms. In contrast, a steady decrease in *E. coli* C157 OmpC mutant cell concentration was calculated across days 3 to 5, with a 65% drop in cfu in the 3 and 5 day-old biofilms. Further, a sharp 63% increase in the concentration of *E. coli* C157 was observed between the 5 and 6 day old biofilms.

Adsorption of T4 to *E. coli* biofilms is not OmpC-dependent. Adsorption was indirectly measured as free-floating phage remaining at 9 minutes post-inoculation and normalized to enumerated *E. coli* cells for each biofilm. The *E. coli* C157 OmpC mutant were originally included as a negative control of adsorption and infection (13). However, significant adsorption of free-floating phage was observed in the *E. coli* C157 biofilms, comparable to the *E. coli* C149 wild-type biofilms of the same age (FIG. 3A).

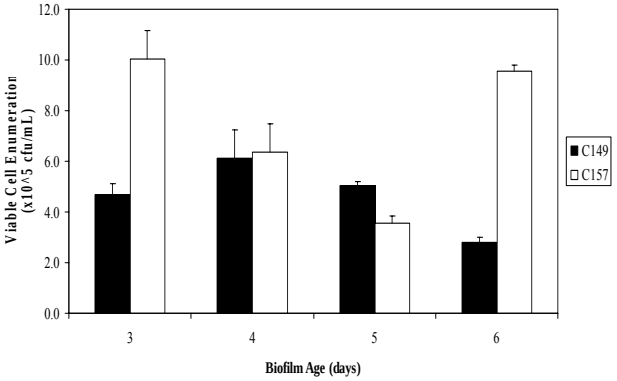

FIG. 2. Effect of the OmpC mutation on the concentration of *E. coli* biofilm cells. Error bars derive from mean absolute deviance of replicate plates and represent the average of two experiments.

Adsorption of T4 to *E. coli* C149 and C157 biofilms decreases with biofilm age. Both *E. coli* C149 wild-type and *E. coli C157* OmpC mutant 3 and 4 day-old biofilms absorbed more phage than the corresponding 5 and 6 day-old biofilms (FIG. 3A). Increases in free-floating phage were observed between the 4 and 5 day-old biofilms; 1.6-fold and 3.5-fold differences for wild type and OmpC mutant biofilms, respectively. Significantly more free-floating phage were measured in the OmpC mutant than the wild-type 5 day-old biofilms.

T4 adsorption to *E. coli* biofilms does not correlate with T4 progeny production. T4 phage progeny production was measured at 60 minutes post-inoculation as free-floating phage normalized to enumerated *E. coli* cells for each biofilm. Normalized phage production by *E. coli* C149 wild-type cells decreased 71% between 3 and 4 day-old biofilms and increased 2.7-fold between 5 and 6 day-old biofilms (FIG. 3B). This pattern of progeny production correlated with levels of free-floating phage but not

T4 adsorption at 9 minutes post-infection. A similar observation was made for the *E. coli* C157 OmpC mutant biofilms. Normalized phage production increased 20-fold between the 3 and 5 day-old OmpC mutant biofilms, then decreased 93% between the 5 and 6 day-old samples (FIG. 3A). Again, this correlated with levels of free-floating phage but not T4 adsorption at 9 minutes post-infection which is displayed most prominently in the 5 day-old biofilm (FIG. 3A).

***E. coli* C157 biofilm cells are more capable of supporting phage progeny production than *E. coli* C149 biofilm cells.** A comparison of T4 phage released between 9 and 60 minutes to phage absorbed at 9 minutes post-infection illustrates the efficiency of progeny production by each biofilm (FIG. 4). Four day-old *E. coli* C149 wild-type biofilms were 33% as efficient as the corresponding 3 day-old biofilms. This loss of progeny production relative to adsorbed phage was maintained in the 5 day-old biofilm but increased 38% between the 5 and 6 day-old samples. *E. coli* C157 OmpC mutant biofilms exhibited a trend opposite to the wild-type biofilms. Efficiency of T4 progeny production increased steadily 10-fold between the 3 and 5 day-old OmpC mutant samples, then dropped 88% between the 5 and 6 day-old biofilms. Differences between the wild-type and OmpC mutant biofilms varied between days. Wild-type 3 day-old biofilms were 3.4-fold more efficient than the OmpC mutants, but the mutant 5 day-old biofilms were 28-fold more efficient than the wild-type.

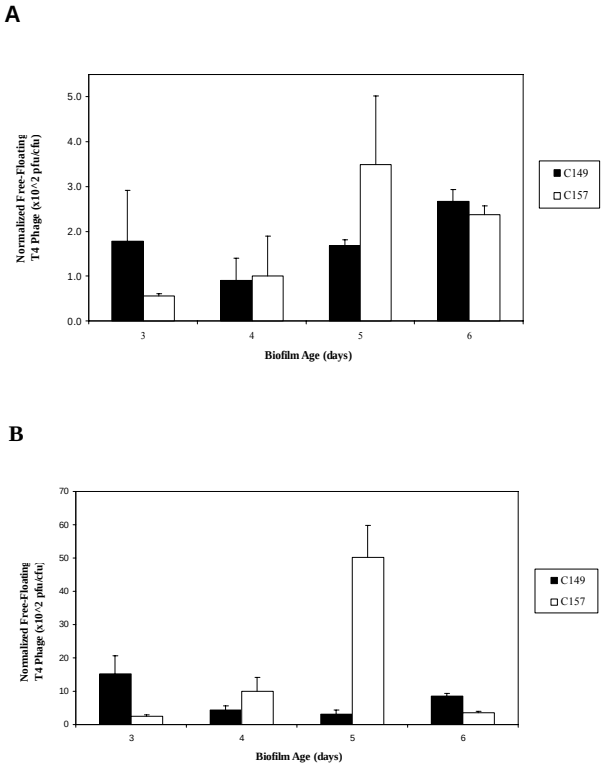

FIG. 3. Effect of the OmpC mutation on T4 attachment **(a)** T4 adsorption to *E. coli* biofilms as represented by free-floating phage at 9 minutes post-infection. **(b)** T4 progeny by 60 minutes post-infection of *E. coli* biofilms, as represented by free-floating phage. Biofilms were inoculated with 2.5 x 10^5 pfu in 5 ml clarified biofilm supernatant and incubated at room temperature on a tube roller, 23 rpm, and samples were taken from the same tubes at 9 minutes and 60 minutes post-infection. Phage values were normalized to biofilm cell counts. Error bars derive from the mean absolute deviations of phage plaque and biofilm cell enumeration replicates and represent the average of two replicates.

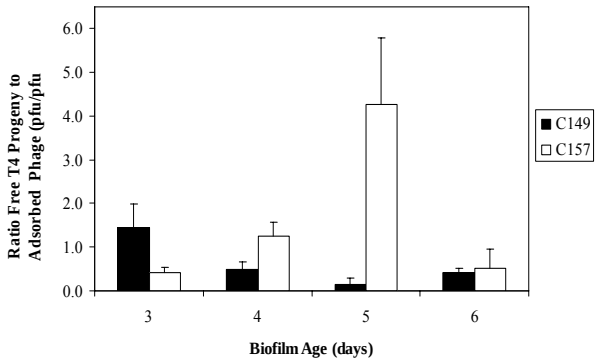

FIG. 4. Efficiency of T4 progeny production by *E. coli* biofilms. Biofilms were inoculated with 2.5 x 10^5 pfu in 5 ml and incubated at room temperature on a tube roller, 23 rpm. Free phage progeny were considered the difference in free phage between 60 and 9 minutes post-infection; adsorbed phage were calculated as the difference between inoculating phage and free phage at 9 minutes post-infection. Error bars derive from mean absolute deviation of replicate plates and represent the average of two experiments.

DISCUSSION

T4 bacteriophage adsorption to biofilms was found to be highest for four day-old biofilms. This supports the idea that OmpC may be over-expressed starting at day four. However, unlike Chan *et al.*, we did not observe a significant increase in T4 bacteriophage progeny production from a four day-old *E. coli* biofilm (FIG. 3B). This may be due to the use of different *E. coli* strains. Chan *et al.* used *E. coli* ZK126 for their biofilm experiments while we used *E. coli* strain C149 as our wild-type. We chose to use this strain because other than having no mutation in its *ompC* gene, the *E. coli* C149 strain is isogenic to the OmpC mutant *E. coli* C157 strain. However, *E. coli* C149 has more mutations than *E. coli* ZK126 and some of the genes mutated were found to be involved in amino acid biosynthesis as shown from the information found at the Encyclopedia of *Escherichia coli* K-12 Genes and Metabolism website (http://ecocyc.org/). This might decrease the fitness of C149 cells compared to

ZK126 cells in stressed environment like a biofilm, and cause genes regulated by the stress response sigma factor, *rpoS*, to be induced (8). This will be discussed in further detail at a later point.

We expected to observe significantly less T4 phage adsorption to biofilms of the *E. coli* C157 OmpC mutant compared to the *E. coli* C149 wild-type biofilms on each day (FIG. 3A) because adsorption to *E. coli* is known to occur mostly through both lipopolysaccharide (LPS) and OmpC (15). However, similar levels of phage adsorption were detected for C157 OmpC mutant and C149 wild-type biofilms (FIG. 3A). In fact, C157 three day-old biofilms displayed more adsorption than C149 three day-old biofilms. Due to the absence of OmpC, this adsorption could initially be attributed to T4 adsorbing solely to LPS. This is possible since *lpxC*, a gene encoding a protein associated with lipid A biosynthesis, was shown to be up-regulated in biofilm formation (11). However, it cannot be confirmed that this directly increases LPS levels despite the fact that lipid A is a component of LPS. Therefore other outer membrane proteins expressed by the *E. coli* C157 OmpC mutant biofilms could potentially be used by T4 bacteriophage for binding purposes.

The changes observed in T4 phage adsorption to C157 and C149 biofilms of different ages (FIG. 3A) suggest that biofilms are adjusting to the stresses brought on by the environment. This can be expected because biofilms are composed of cells of different viabilities that must co-ordinate with each other in order to maintain stationary-phase growth (9). We recognized that adsorption can occur on both viable and non-viable cells. Therefore, decreased concentrations of non-viable cells could make it appear that there is less adsorption occurring, vice versa. However, attempts to enumerate both viable and non-viable cells in the biofilms with crystal violet were not successful due to technical difficulties. Nonetheless, it is generally thought that as biofilms mature, non-viable cells continuously fall off to expose new receptors for T4 adsorption (12). Therefore we believed that this comparison is still valid.

As described earlier, where an increase in adsorption is observed, it does not appear to correlate with an increase in progeny production at 60 minutes for biofilms of the same age (FIG. 3A and FIG. 3B). Firstly, this suggests that the inability to produce phage progeny is not due to any problems with initial adsorption. Instead, the viral replication steps following adsorption are responsible for this trend because the phage had no problems binding to both wild-type and OmpC mutant biofilms. After reversible binding with LPS or irreversible binding with OmpC (2, 10), there may be problems with any of the subsequent steps in the T4 replication resulting in decreased progeny production or in some cases there may be absorption but no replication whatsoever. These steps include penetration – the injection of phage DNA into the host cell following irreversible binding, synthesis of viral enzymes, proteins and DNA, followed by the formation of new phage particles, and finally the lysis of the host cells with the release of progeny (2, 10). However, further experimentation will be required to determine at which step viral replication is halted in *E. coli* biofilm cells.

A second reason that T4 adsorption to biofilms do not correlate with progeny production is that many stress response systems and their effectors are activated during the development and maturation of *E. coli* biofilms (4). These include the general stress response gene (*rpoS*), the heat shock or oxidative stress-induced sigma factor E (*rpoE*), the *psp* phage shock proteins, as well as genes involved in the SOS response and DNA repair systems (4, 5). Hence, the expression of these stress responses in *E. coli* biofilm cells suggest that expression of viral enzymes, proteins and DNA replication may be inhibited to some extent, leading to the observation that increased absorption does not correlate with increased progeny production.

A third reason that only applies to the C157 OmpC mutant biofilms is that OmpC plays a role in the production of membrane components. For example, OmpC can regulate the stability of RseA, an anti-σ inhibitor, which in turn increases the activity of Sigma E, σ^E (1, 14). Sigma E then leads to the production of various membrane components in times of stress (14), as expected from biofilms. Due to the lack of OmpC, these other membrane components that act as receptors may be down-regulated in C157 and therefore reveal decreased phage adsorption in the 5 day-old biofilm (FIG. 3A). However, at the same time, since OmpC mutant *E. coli* cells display increased motility (14), biofilm formation might be less efficient. This can decrease the amount of exopolysaccharide present in the extracellular matrices of a biofilm that would otherwise trap T4 phage released after progeny production. As a result, this can lead to the detection of higher titres of free phage released into the media. Together they can potentially explain the observed increase in phage progeny production despite lower levels of T4 phage adsorption.

In the three reasons mentioned above, we assumed that all free-floating phage in figure 3A was phage that could not attached to the biofilms. However, high levels of both free-floating phage and progeny at 9 and 60 minutes post-infection

respectively, could mean that the T4 phage is attaching poorly to the biofilms. At 9 minutes this would reveal high levels of free-floating phage. And since this same biofilm does not allow efficient attachment, progeny production from phage that successfully attached to cells at 9 minutes will remain in the supernatant instead of attaching to adherent biofilm cells. Similarly low levels of both free-floating phage and progeny could mean that T4 phage attachment to the biofilms is efficient. As a result, progeny released would immediately attach to adherent biofilm cells or remain trapped in the extracellular matrices of the biofilm instead of being detected in the supernatant. Therefore, the efficiency of attachment to biofilms of different ages could explain why progeny production at 60 minutes correlates with free-floating phage instead of T4 adsorption at 9 minutes post-infection.

E. coli C149 wild-type and C157 OmpC mutant biofilms displayed opposite trends in the efficiency of progeny production (FIG. 4). This can be explained by the different gene expression profiles already present between the C149 wild-type and C157 OmpC mutant cells prior to and during biofilm formation. For example, because C157 *E. coli* does not have functional OmpC, they should be already adapted to survive and multiply even without this major porin. Also, they did not display delayed growth from our culturing observations, hence they should have also up-regulated compensatory genes such as OmpF (3). It could be hypothesized that C157 OmpC mutant cells are already primed to respond to stressful environments, which are often encountered in biofilms. While C149 wild-type cells are not primed and might display stress as the biofilms mature, this stress might account for the decreased efficiency of T4 phage progeny production (FIG. 4). Regardless of the mechanism, our results suggest that C157 and C149 *E. coli* biofilms have different capacities to support phage production. More specifically, C157 OmpC mutant cells may have the capacity to support more phage production than C149 despite having less phage adsorption.

Although we obtained T4 adsorption results that reflected the T4 phage progeny production in the *E. coli* K12 biofilms observed by Chan *et al*., we could not replicate the phage progeny production results. Therefore we conclude that phage adsorption does not correlate with phage progeny production by C149 and C157 *E. coli* biofilms.

FUTURE EXPERIMENTS

Since we did not observe a correlation between T4 phage progeny release and initial phage adsorption, phage DNA injection, transcription and translation of phage genes, or phage packaging could account for the pattern of phage progeny production as the biofilm aged. Also, it is unknown from our results whether initial phage adsorption consistently led to the irreversible binding of phage to the biofilms and subsequent phage progeny production, or if a significant amount of the binding was reversible. Hence, the amount of reversible and irreversible binding of T4 bacteriophage to biofilms of wild-type *E. coli* and *E. coli ompC* mutants should be determined to further assess the binding of T4 phage to the different biofilms. This could be done by performing an overlay assay with chloroform-treated resuspended biofilm pellets that were incubated shortly with T4 bacteriophage to determine the titer of the reversibly bound phage. In another experiment, phage DNA injection may be visualized in host *E. coli* by fluorescent in situ hybridization after adsorption by using a probe specific for T4 bacteriophage DNA.

Furthermore, the level of cellular stress may play a role in the pattern of phage production as biofilms age. In the future, the level of stress in OmpC mutant and wild-type *E. coli* biofilms could be tested by measuring and comparing the level of RpoS protein, the global stress regulator. A possible method would involve immunoblotting with an anti-RpoS antibody. Finally, the basis for the decrease in biofilm cell concentration as they age should be determined because this will reveal whether the decrease in biofilm cell concentration is due to cell detachment from the biofilm into the clarified media or due to biofilm cell death. This could be determined by measuring the amount of cell detachment from biofilms into the clarified media. Enumeration of detached biofilm cells could be measured by sampling the clarified media at different time points after treatment of biofilms with the clarified media and spread-plating the samples onto LB agar plates.

ACKNOWLEDGEMENTS

Our work was supported by the Microbiology and Immunology Department of The University of British Columbia (Vancouver, BC, Canada), which also supplied the bacterial strains and phage. We extend thanks to Lando Robillo and Technical Support of the department for providing TSG bottom agar plates, molten agar tubes, advice and encouragement. Our gratitude also goes to Shannon Hui, Tina Lin, Antoinette Cheung, and Carrie Wang for providing additional TSG bottom agar plates. We especially wish to thank Dr. William Ramey and Shaan Gellatly for their knowledge and patient guidance throughout the experiments.

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