Cell elongation induced by *ftsZ* antisense RNA expression in *Escherichia coli* strain DH5α increases T4 bacteriophage adsorbed per cell

Derrick Chong, Emmanuel Garrovillas, Alice Huang, Nathan Naidu

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY Adsorption is a fundamental step in the bacteriophage life cycle that results in the irreversible attachment to external asperities on the cell surface of *Escherichia coli*, allowing for the eventual injection of the viral genome for replication. Previous research has demonstrated that *E. coli* grown in rich media were larger and adsorbed more T4 bacteriophage than cells that were grown in less carbon-rich media. However, the direct effect of modulating cell size on phage adsorption is unclear. To investigate the relationship between cell size and T4 phage adsorbed per cell, we used antisense inhibition of *ftsZ*, an essential cell division gene, in *E. coli* strain DH5α, to induce cell elongation. Induction of *ftsZ* antisense RNA resulted in a distinct cell shape morphology that is elongated and filamentous as a consequence of impaired cell division. We quantified T4 bacteriophage adsorption in elongated FtsZ deficient and normal length wildtype *E. coli* using double agar overlay plaque assay. We found that elongated cells adsorbed 16% more phage than wildtype cells. The elongated morphology and filamentation in *E. coli* with decreased *ftsZ* activity was visualized by crystal violet staining and phase contrast microscopy, which is consistent with the idea that T4 bacteriophage adsorption increases with *E. coli* cells that have greater surface area. Whether other methods of inducing elongated growth will increase bacteriophage adsorption, as well as its effects on subsequent steps of the bacteriophage life cycle, remain to be elucidated.

INTRODUCTION

Bacteriophage adsorption on bacterial cells is regulated through physical contact between the host cell surface and bacteriophage receptor-binding motifs (1). Phage adsorption initiates the infection process and is a crucial determinant of host tropism and specificity (2). Adsorption is defined as a three-step process: initial contact, reversible binding, and irreversible attachment (1). The phage must bind to the correct receptor(s) on the host cell surface through specific interactions with the phage tail apparatus during initial contact, before it can attach irreversibly and insert its genome into the host (1, 3-4). Receptors of phage include external asperities on the cell surface, such as lipopolysaccharides (LPS), peptidoglycan, porin proteins, pili, fimbriae, fibrils, and polymeric fringes (5). Adsorption of T4 bacteriophage to *E. coli* K-12 has been demonstrated through binding LPS and outer membrane protein C (OmpC) (6). Previous research has proposed adsorption to either be OmpC-dependent or OmpC-independent (7). Bacteria can modulate phage adsorption to their cells through blocking receptors through conformational or structural changes, such as the loss, modification or masking of a targeted bacterial receptor (2, 8, 9). In response, adaptation of the phage receptor-binding proteins occurs to recognize an unidentified alternative primary receptor (2).

Bacterial cell envelopes are dynamic components which control major cell functions varying from cell growth and division, morphology, and the antigenic properties of bacteria (10). Previous research on cell shape have investigated the altered morphology of *E. coli* when induced by general forms of stress such as starvation and osmotic shock, and found rounding and length reduction of the characteristic rod shape (11). Spherical rounding due to environmental stress is adopted by membranous cells to conserve and minimize energy (12). Additionally, Choi et al. previously established the relationship between cell size and T4 phage burst size, which is defined by the number of phage progeny released from

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Address correspondence to: https://jemi.microbiology.ubc.ca/

infected cells after a single round of infection. In accordance with the principle that a single bacterium can adsorb many phage particles, Choi et al found that larger cells have an increased burst size due to a higher number of phage present during the initial infection (13). In addition to cell size, the observed increase in burst size was also attributed to increased protein synthesizing machinery (13). However, the distinct relationship between cell size and the number of phage progeny produced depends on the assumption that larger cells have greater T4 bacteriophage adsorption. Consequently, we aim to further test this conjecture by investigating bacteriophage adsorption as a function of cell shape and size.

To examine bacteriophage adsorption, we modulated cell shape using two *E. coli* strains that varied in length and size. The absence of an essential division protein in *E. coli* prevents cell division and generates an elongated bacterial cell (14). FtsZ is an essential tubulin homologue that acts as a central scaffold in recruiting and maintaining the Z-ring in *E. coli* (9). Similar to tubulin, FtsZ is a GTPase that polymerizes into dynamic protofilaments in the presence of GTP, tethering to the inner membrane and assembles to form the Z-ring (15). Upon assembly, the Z-ring recruits proteins required for the progression and completion of cytokinesis, ultimately generating a constrictive force that pulls the cytoplasmic membrane inward (16-17). Deletions in genes involved in assembly of the Z-ring lead to elongated cells, abnormal Z-rings, and irregular septum morphologies; bacterial cell shape is modulated through the expression of *ftsZ* (18). Electron micrographs of isogenic cell division mutants of the Fts family, including FtsZ, have double their mass (19). Therefore, previous observations of elongated *E. coli* because of impaired cell division allows us to investigate the relationship between cell shape and bacteriophage adsorption.

To generate elongated cells and compare the parameter of cell shape on phage adsorption, antisense plasmid technology was used to knock down expression of *ftsZ* at the translation level. DNA segments encoding antisense RNA (asRNA) were designed so that the resulting asRNA would bind complementary to *ftsZ* mRNA regions (20). Through sharing extensive sequence complementarity, asRNA can inhibit mRNA from ribosome binding and gene translation, often resulting in mRNA degradation (20).

Previous work has shown that *E. coli* cells growing faster in rich media adsorb more bacteriophage, yet no imaging was done to determine cell length (21). It has been previously established that faster growing cells are larger, however it remains to be confirmed that larger cell size does truly increase adsorption (22). Therefore, the purpose of our study was to modulate cell size and enumerate the effect of T4 bacteriophage adsorbed per cell. We hypothesized that by inducing elongation with *ftsZ* asRNA, the percentage of bacteriophage adsorbed would be greater than *E. coli* that was not elongated.

METHODS AND MATERIALS

Bacterial and Bacteriophage Strains. *E. coli* strains DH5α, DEAN-pZ, and DEAN-pHN678 (Table S1), and T4 bacteriophage, were obtained from the lab stocks of the Department of Microbiology and Immunology at the University of British Columbia.

IPTG Induction and staining. DEAN-pZ and DEAN-pHN678 were grown in LB broth with 50 μg/mL chloramphenicol at 37°C overnight. Cultures were diluted 1:100 and incubated again for approximately 3 hours. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added at concentrations of 1 mM, 10 mM, and 20 mM. A crystal violet stain on heat fixed smears was performed to visualize filamentous growth approximately 3 hours post-induction at 37°C.

T4 Bacteriophage Propagation. *E. coli* BW25113 was grown in LB broth at 37°C overnight. Culture was diluted 1:6 in fresh LB broth and mixed with a picked plaque of T4 bacteriophage. The mixture was incubated at 37°C and shaken at 200 RPM for 6 hours. Chloroform was then added to the mixture to kill any remaining cells and centrifuged at 5000 RPM for 5 minutes to pellet cell debris. Supernatant containing T4 bacteriophage was removed and stored at 4°C.

Enumeration of T4 Bacteriophage Titer. T4 bacteriophage was serial diluted from 10^{-1} to 10^{-8} in a 96-well plate with PBS. Underlay agar was prepared with tryptone, yeast extract,

NaCl, and 15 g/L agar. Soft overlay agar was prepared using the same components but instead with 5 g/L agar. An overnight culture of *E. coli* BW25113 was refreshed by performing a 1:100 in fresh LB and incubated for 3 hours at 37°C with shaking at 200 RPM. Soft overlay agar was supplemented with 1 mM $CaCl_2$ to facilitate bacteriophage assembly and warmed to 60°C in 3 mL aliquots. To each aliquot of soft agar, 100 µL of refreshed culture was added. Tubes were quickly mixed to prevent solidification in the tube and were poured onto underlay agar plates supplemented with 1 mM $CaCl_2$. Plates were dried for 30 minutes and then 5 µL of each T4 bacteriophage dilution was spotted onto the plates using a multichannel pipette. Spots were left to dry before inverting the plates and incubating at 37°C overnight. Plaques were counted the following morning.

PCR and Gel Electrophoresis. Polymerase Chain Reaction (PCR) was carried out using *Taq* DNA polymerase (New England BioLabs) according to the manufacturer's protocol. Primers designed by Chiu et al. (23) to amplify *gp23* and *gp10a* were used to confirm whether the T4 bacteriophage stock was contaminated. PCR products were resolved on a 1% agarose gel in 1X TAE with a NEB 1 kb Plus DNA Ladder and visualized using ethidium bromide.

T4 Bacteriophage Adsorption Assay. The adsorption assay protocol used was modified from Dimou et al. (24) and Kropinski (25). DEAN-pZ and DEAN-pHN678 cultures were induced with 20 mM of IPTG (see above) and diluted 1:50 in LB. Cells were visualized using crystal violet to ensure filamentous and non-filamentous growth for DEAN-pZ and DEAN-pHN678, respectively. Each culture was treated with $2x10^4$ plaque-forming units (PFU) of T4 bacteriophage and incubated at 37°C with shaking at 200 RPM for 5 minutes. Chloroform was then added to each tube, vortexed for 10 seconds, and incubated on ice for 10 minutes to lyse all cells. Lysates were centrifuged and the phage-containing supernatant was collected, avoiding the chloroform-containing bottom layer. A 1/5 and 1/10 dilution in phosphate buffer saline (PBS) of each supernatant was prepared while 4 mL aliquots of soft overlay agar containing 1mM $CaCl_2$ were pre-warmed to 60°C. In triplicate, each supernatant and their corresponding dilutions were added to the aliquots of soft overlay agar, followed by an aliquot of refreshed *E. coli* DH5α overnight culture, previously described in the bacteriophage enumeration. The mixture was mixed gently by hand and poured onto underlay agar plates supplemented with 1 mM $CaCl_2$. Simultaneously during this, serial dilutions of the refreshed DEAN-pZ and DEAN-pH678 cultures were prepared ranging from 10^{-1} to 10^{-12}. Dilutions 10^{-4} to 10^{-9} for both cultures were spread on LB plates in duplicate and incubated at 37°C overnight for colony-forming unit (CFU) determination. Plates were left to dry for an hour before incubating at 37°C overnight. Plaques and colonies were counted the following morning.

Calculations for %PFU and normalization. The percentage of T4 bacteriophage that adsorbed per cell was calculated as follows:

$$Phage\ Adsorbed\ (\%\ PFU)\ = \frac{PFU_{initial}\ -\ PFU_{final}}{PFU_{initial}} \times 100\%$$

$$CFU\ Normalization\ Factor\ = \frac{CFU_{DEAN-pZ}}{CFU_{DEAN-pHN678}} = 0.914$$

$$\%PFU_{DEAN-pHN678(normalized)} = 0.914\ *\ \%PFU_{DEAN-pHN678}$$

CFU for DEAN-pZ and DEAN-pHN678 was counted to normalize the percentage of adsorbed T4 bacteriophage. There were 8.6% less CFU in DEAN-pZ than DEAN-pHN678, therefore a normalization factor of 0.914 was determined and used to account for less DEAN-pZ cells present during the adsorption assay. $PFU_{initial}$ represents the $2x10^4$ PFU added into each culture. PFU_{final} represents the free phage in the supernatant plated in a double agar overlay plaque assay.

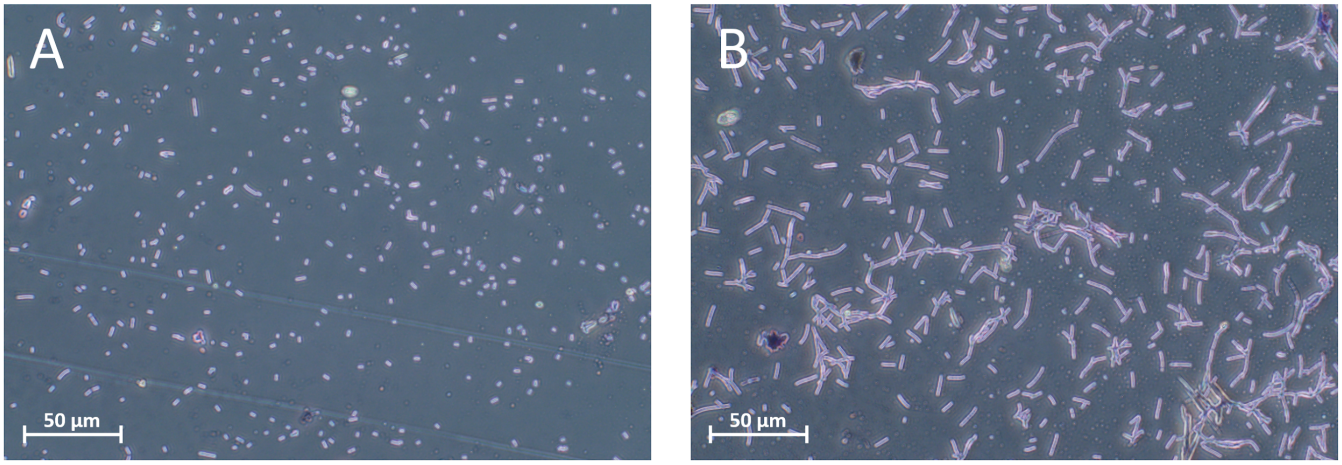

FIG. 1 Induction of DEAN-pZ with IPTG confirms filamentous growth. Crystal violet stains of cultures after a 3-hour incubation with 20 mM IPTG for (A) DEAN-pHN678 and (B) DEAN-pZ to confirm expected cell shape in both strains. Heat-fixed smears were flooded with crystal violet and incubated for 1 minute and then visualized by phase contrast microscopy at 40X magnification.

RESULTS

Phase contrast microscopy shows elongation of *E. coli* DH5α expressing *ftsZ* antisense RNA. To study the effects of cell elongation on the adsorption of T4 bacteriophage, we acquired the DEAN-pZ plasmid encoding a *ftsZ* asRNA to inhibit *ftsZ* translation, as well as its empty vector control, DEAN-pHN678. DEAN-pZ encodes an IPTG inducible asRNA that targets the ribosomal binding site (RBS) of *ftsZ* mRNA, preventing ribosomal binding and translation of FtsZ (20). Depletion of FtsZ prevents the Z-ring formation during cell division, resulting in filamentous-like growth in *E. coli* (26). To verify the identity of DEAN-pHN678 and DEAN-pZ, crystal violet stains of heat-fixed cells were performed after a 3 hour induction with 20 mM IPTG to observe cell shape. DEAN-pHN678, the empty vector control not encoding an *ftsZ* asRNA, did not show any cell elongation after IPTG induction (Fig. 1A). Crystal violet stained DEAN-pZ elongated by a factor of 3.25 compared to DEAN-PHN678 after IPTG induction (Fig. 1B). These results confirm that DEAN-pZ elongated following *ftsZ* asRNA induction, while DEAN-pHN678 displayed normal cell morphology.

Elongation of *E. coli* DH5α increases adsorbed T4 bacteriophage per cell. The difference in T4 bacteriophage adsoprtion between the elongated DEAN-pZ strain and the DEAN-pHN678 control was measured using a double overlay agar assay. T4 bacteriophage was titered and verified by PCR amplification of *gp23* of the T4 bacteriophage genome (Fig. S1). Log-phase *E coli* strains DEAN-pHN678 and DEAN-pZ were induced for 3 hours with 20 mM IPTG, and then treated with $2x10^4$ PFU of T4 bacteriophage for 5 minutes. Following induction and bacteriophage treatment, cells were immediately incubated with chloroform to kill all remaining bacteria, including cells with injected phage-genome material, leaving the unadsorbed bacteriophage intact (25). The mixture was vortexed and centrifuged to separate the phage-containing supernatant from the chloroform, which was diluted 1/5 and 1/10 in PBS. Undiluted, 1/5, and 1/10 dilutions of the phage supernatant were then plated in triplicate with wild-type *E. coli* DH5α to determine the number of PFU produced. CFU was then determined by plating serial dilutions of the DEAN-pHN678 and DEAN-pZ cultures used for the T4 bacteriophage adsorption assay. After determining the PFU of free phage and CFU from the DEAN-pHN678 and DEAN-pZ cultures, the amount of adsorbed T4 bacteriophage was determined. The data was then normalized to equivalent CFU values and expressed as the percentage of adsorbed phage in respect to the initial amount of T4 phage used to infect both strains. DEAN-pHN678 served as the control, which did not express asRNA of *ftsZ*, and adsorbed 16% less T4 bacteriophage than the elongated DEAN-pZ. This suggests that induction of filamentous growth in *E. coli* DH5α increases the number of phage adsorbed per cell.

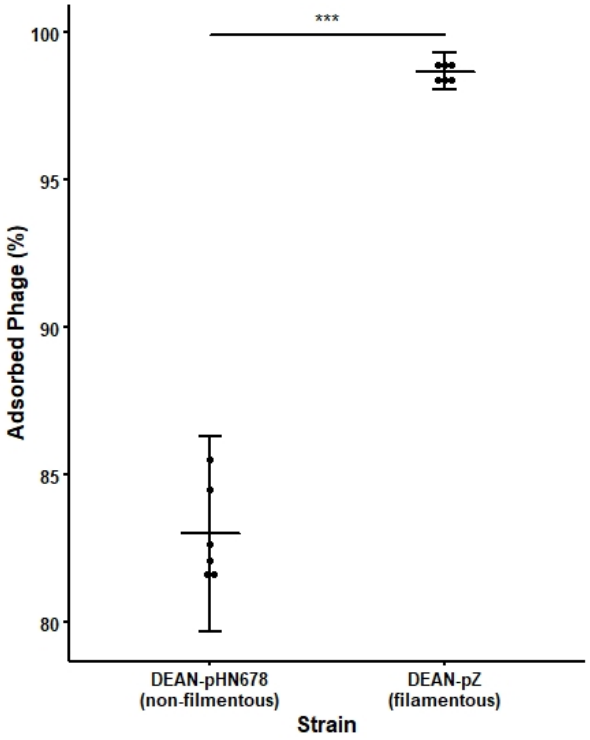

FIG. 2 Filamentous DEAN-pZ adsorbs more T4 bacteriophage than non-filamentous DEAN-pHN678. DEAN-pHN678 and DEAN-pZ were treated with $2x10^4$ PFU of T4 bacteriophage for 5 minutes, after which the free phage supernatants were plated with *E. coli* DH5α in soft overlay agar. The data was normalized to the CFU present during the T4 bacteriophage infection, and represented as the percentage of adsorbed phage in relation to the amount of phage initially used for infection. Data represented as mean ± 2S.D. (n=6). *** $p < 0.0001$.

DISCUSSION

In this study, we investigated cell length as a determinant of T4 bacteriophage adsorption. We hypothesized that cells produced by inhibition of cell division, which are likely longer but identical daughter cells fused together, will have more surface area containing phage receptors to bind phage. To investigate this, we deployed an adapted version of a phage adsorption assay previously described by Dimou et al. (24). The assay involved the induction of filamentous growth with subsequent treatment with T4 bacteriophage, followed by treatment with chloroform, allowing us to quantify free phage present in the media and calculate the percentage of adsorbed phage. Simultaneous plating of CFU and PFU was performed to standardize the number of adsorbed phage to live filamentous and live non-filamentous cells. A five-minute phage treatment period was chosen because it was previously shown that approximately 50% of T4 bacteriophage adsorb to *E. coli* strain MG1655 after 5 minutes of incubation (24). It was important to identify a time period that was long enough to see a difference in phage adsorption between conditions, but short enough to prevent all free phage from adsorbing. Lastly, a 3-hour induction time was performed on log-phase cultures due to our preliminary observations in which cultures are non-viable if they are directly inoculated into IPTG-containing media. We speculate that this is because they do not divide and therefore eventually lyse once the cell surface area to volume ratio is out of proportion. This 3-hour induction time was also an ideal time where we noticed drastic changes in cell length and we did not proceed with longer induction to prevent the generation of dead cells that may adsorb phage. Dead cells would not contribute to the CFU and may skew our calculations by contributing to adsorbing phage.

Longer cells adsorb more T4 bacteriophage. We observed that the longer filamentous cells adsorb 16% more T4 bacteriophage. Since FtsZ depletion results in cells that undergo the replication cycle, but fail to divide by creating a septum, these cells will be longer, but are hypothetically multiple identical daughter cells fused together due (18). Therefore, we predicted that the number of phage receptors on a singular cell will proportionally increase with the cell length. Phage receptors include LPS and OmpC, the outer membrane protein involved with osmoregulation (6). Osmoregulators are critically important for basic cell function, and are particularly regulated to accommodate for cell volume (27). Therefore, it

is possible that an elongated cell from FtsZ depletion likely has more surface area for phage receptors like OmpC, or expresses more OmpC to compensate for the cell volume and will consequently adsorb more phage.

Additionally, previous work has also correlated increased cell size to more T4 phage adsorption through different methods (21-22). Hadas et al. observed that cells growing with high growth rates are longer than cells with slower growth rates (21). Hadas et al. also observed a similar trend when they pre-treated cells with penicillin, a β-lactam antibiotic that can inhibit cell division at low concentrations (21). However, the authors did not report that they visualized their cells prior to treatment with bacteriophage, so an increase in cell size was not confirmed (21). Furthermore, the change in cell physiology and cell viability by nutrient change and antibiotic addition was not investigated. Since changes to media composition can change the osmolarity of the media, this may also influence expression of *ompR*, which responds to changes in osmolarity and regulates *ompC* expression (28). We addressed these study limitations by inducing cell elongation using *ftsZ* asRNA to prevent cell division.

FtsZ depletion may affect phage adsorption in multiple ways. Our method of elongating cells was chosen under the assumption that cells deficient in FtsZ would elongate by generating nearly identical, longer and undivided daughter cell equivalents. Therefore, a single cell will have more receptors for binding phage. Despite the longer FtsZ depleted cells adsorbing more phage and agreeing with our original hypothesis, we were curious if FtsZ depletion was consequential to cell physiology beyond division. Recent studies have shown that FtsZ deprived *E. coli* accumulate defects in their physiology including nucleoid accumulation, membrane destabilization, and increased expression of transcriptional regulators, *hns* and *sptA* (29). Interestingly, the transcriptional regulators *hns* and *sptA* control expression patterns of outer membrane proteins including phage receptor OmpC (30). Together, these suggest that the depletion of FtsZ may have pleiotropic effects on cellular physiology, which may explain why we do not see a proportional increase of phage adsoprtion on elongated FtsZ depleted cells. However, it is not known if one or more of these effects contributed to the increased phage adsorption we observed in the FtsZ depleted cells. Investigating these effects may also explain why phage adsorption only increased by 16% while cell surface area increased 325% in FtsZ depleted cells.

Conclusions Our results show that the elongated *E. coli* DH5α depleted of FtsZ adsorb more T4 bacteriophage than shorter cells with functioning FtsZ. However, the consequences of FtsZ depletion on other aspects of cellular physiology that may contribute to phage adsoprtion, remains to be elucidated.

Future Directions We demonstrate that adsorption, the first part of the T4 bacteriophage infectious life cycle, is affected by *ftsZ* and possibly cell length and size. Further investigation on how both *ftsZ* and cell length may affect the full infectious T4 bacteriophage life cycle may help elucidate the role of different cell sizes in an environment. It is possible that larger bacteria may adsorb more phage and may even be advantageous for cells to be smaller to avoid phage adsorption. Depleting FtsZ and performing growth curves and plaque assays may reveal implications on host fitness or phage success in terms of burst size. Additionally, DEAN-pZ may be used to elucidate the relationship between *ftsZ* and major phage receptors, OmpC and lipopolysaccharide, by determining expression of these receptors changes upon ftsZ depletion. Since previous work has suggested that FtsZ depletion affects transcriptional regulators and membrane integrity, these parameters may be investigated to determine the specific mechanism behind increased phage adsorption in DEAN-pZ.

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