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Exploring K30 capsule production by *E. coli* E69 as a potential mechanism of resistance to T4 bacteriophage infection

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SUMMARY Bacterial capsules are extracellular structures located on the outer cell wall, and capsule production has been shown to both promote and inhibit bacteriophage susceptibility, with specific interactions seemingly dependent on the capsule serotype and phage used. Understanding the relationship between bacterial characteristics and phage resistance is valuable to aid in the improvement of phage therapy, which is used to treat illnesses caused by pathogenic bacteria. The aim of our experiment was to investigate if capsule production inhibited T4 bacteriophage infection in group I capsule-producing K-30 strain *Escherichia coli* E69. The K-12 mutant CWG655 has a triple chromosomal deletion of *wza-wzb-wzc*, encoding for proteins responsible for surface expression of capsular polysaccharides. Based on prior research indicating that T4 resistance was dependent on the presence of K5 capsule in *E. coli* strain Nissle 1917, it was hypothesized that the production of capsule in E69 would confer resistance to T4 bacteriophage, while CWG655 would be susceptible to phage infection. A preliminary experiment testing infectivity was carried out using a double agar plaque assay, with plaque formation observed in CWG655 but not in wild-type E69. This may indicate that the capsular polysaccharide (CPS) production in wild-type E69 conferred resistance to T4. Further experiments are needed to confirm these initial findings. The mechanism of T4 phage resistance and interactions of CWG655 with other bacteriophages could also be investigated to further understand bacteriophage resistance dynamics for phage therapy.

INTRODUCTION

Bacterial capsules are hydrated polysaccharide structures covering the outer cell wall in Gram-negative and Gram-positive bacteria, and are composed of individual sugar moieties linked by glycosidic bonds. Certain protein-carbohydrate variations have been described, and the structure remains highly variable (1). Due to its extracellular nature, CPS can play a major role in bacterial virulence and colonization in pathogenic strains by preventing desiccation, influencing phagocytotic success, and decreasing complement-mediated lysis (2). Capsules have a considerable impact on immune evasion and extracellular defense. However, CPS production is energy expensive, and the activation of the pathway is dependent upon the detection of environmental stressors, thus regulation of CPS is influenced by multiple genes (2). Previous research in knockout strain CWG655 demonstrated that the presence of CPS resulted in decreased susceptibility to macrolides, a class of antibiotics (1). More recently, the relationship between capsule production and bacteriophage resistance has gained interest as a novel field of research with potential applications in phage therapy. Whether capsule presence confers phage resistance or susceptibility appears to depend on the type of phage and the capsular serotype, with some data suggesting decreased capsule production is beneficial in phage-resistant mutants (5), while others illustrate capsular blockage of adhesion towards phages such as T7 (6). Certain phages such as PNJ1809-36 are serotype specific (7), but further investigation of capsule-mediated resistance is needed. This study aims to employ K-12 *E. coli* CWG655 and K-30 *E. coli* E69 to assess whether capsule production in E69 confers T4 bacteriophage resistance.

Escherichia coli CWG655 has a complete gene knockout of the *wzy* gene cassette involved in group I capsule biosynthesis. The 3 genes *wza, wzb* and *wzc* encode for several proteins with distinct functions related to surface expression of capsular polysaccharide. Wza is an outer membrane protein which polymerizes to allow translocation of capsule subunits

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while Wzb is an integral inner membrane protein with tyrosine autokinase activity that facilitates capsule polymerization (1). Wzc is a cytoplasmic protein and is the cognate phosphatase of Wzb (1). Ultimately, deletion of all three components renders *E. coli* strain CWG655 unable to synthesize capsular polysaccharides, which possibly accounts for the observed differences in resistance. A previous study by Soundararajan et al. 2019 (2) showed that a capsule-producing *E. coli* strain, cell-free supernatant, and its isogenic capsule knockout strain were all capable of T4 phage inactivation, indicating that T4 bacteriophage resistance occurs through multiple attachment-independent defenses. Presence of capsule-independent resistance mechanisms may explain the inconsistent results observed for T4 bacteriophage resistance of E69 and CWG655 from the plaque and attempted LB broth assays.

Escherichia virus T4 is a species of bacteriophage belonging to the Myoviridae family that infects *E. coli* (8). Current literature suggests that T4 entry into host cells is a two-step process, consisting of reversible attachment of T4 long-tail fibers, followed by irreversible binding by short-tail fibers to host receptors, facilitating entry of the viral genome (9). The intricate method of infection involves highly efficient contractile-injection machinery (9). Initially, long tail fibers bind the host *E. coli* cell reversibly, followed by a conformational change in the baseplate and irreversible binding of the short fibers to the cell. Next, the sheath contracts to allow the tip of the tail tube to descend, then the capsid and tail tube rotate, allowing the needlelike tip of the phage to pierce the cell membrane. There are further individual sub-steps involved in the phage tip crossing the membrane, including lysosomal activity of the needle, which degrades peptidoglycan (10). The genome injection process relies on the balance between two energetic processes; the energy stored in the contractile sheath, used to fuel the injection itself, and the energy of the dissipation mechanisms (2). The complicated process of the needle-like tip crossing the cell membrane and the process energetics contribute to the difficulty in predicting resistance mechanisms.

As a preliminary study, we employed double agar overlay plaque assays to evaluate whether capsule production contributes to T4 bacteriophage resistance. We demonstrated that our capsule producing strain, E69, was resistant to T4 infection whereas the capsule KO strain, CWG655, was susceptible; this suggests that capsule presence may confer resistance to T4 infection.

METHODS AND MATERIALS

Bacterial and bacteriophage strains. *E. coli* K-30 E69, *E. coli* K-12 CWG655 with triple chromosomal deletion of *wza-wzb-wzc*, responsible for surface expression of capsule (1), *E. coli* BL21, *E. coli* DH5α, and T4 bacteriophage strains were obtained from the Department of Microbiology and Immunology at the University of British Columbia. Synthesis of the CWG655 strain is described in the discussion of PCR confirmation of the strains.

TABLE. 1 List of strains used for experiments

Strain Name	Purpose
BL21	Susceptible to T4 phage infection; used for T4 phage propagation, stock preparation and T4 viral titer quantification.
DH5α	Resistant to T4 phage infection: used as a positive control for T4 phage resistance in infectivity assays.
E69	Capsule-producing: WT of CWG655Δ without wzy gene cassette. Tested for susceptibility/ resistance to bacteriophage infection
CWG655	Capsule-deficient due to wzy gene cassette. Used to isolate the effect of capsule on bacteriophage resistance.

Confirmation of bacteriophage and bacterial strain identity via PCR and gel electrophoresis. PCR was performed on T4 bacteriophage stock to amplify *gp23* using Platinum Taq DNA polymerase and primers T4 gp23 F and R (11) diluted down to 10uM. The reaction was optimized with an annealing temperature of 52℃ for 20s, and further Thermo Cycler conditions and master mix ingredients were used as per the Invitrogen ™

Platinum ™ SuperFi ™ DNA Polymerase User Guide. The amplified product was run on a 75 mL 1% agarose gel alongside the Thermo Fisher 1kb Plus DNA Ladder.

The PCR protocol was adapted from Rana et al. (12). EB6 and EB7 primer stocks specific for the *wzy* gene cassette were obtained from the Microbiology and Immunology Department, UBC at a concentration of 100 uM stock concentrations (Table 2). The primers were diluted to 10 uM working stocks using distilled water and added into the PCR reaction mix as indicated by the Invitrogen ™ Platinum ™ SuperFi ™ DNA Polymerase User Guide. The reaction was optimized with an annealing temperature of 65.1°C. The amplified products were run on a 1% agarose gel alongside the Thermo Scientific O'GeneRuler DNA Ladder.

Sanger sequencing of the gp23 amplicon. After the PCR products were run on a gel and imaged to confirm success of the reaction, they were cleaned with the Invitrogen™ PureLink™ PCR Purification Kit**.** The PCR product was sent for Sanger sequencing to Azenta Life Sciences (formerly Genewiz), with both the forward and reverse primer as pre-defined samples.

TABLE. 2 Primers used for PCR reactions

Primer name	Primer Sequence	Working concentration
EB6 (F)	5'-CCTGGTCAGGGATCCAACAGTCTG-3'	10 uM
EB7 (R)	5'TCGCGGATCCAATTGTTACGA-3'	10 uM
T4 gp23 F	5'-GCCATTACTGGAAGGTGAAGG-3'	10 uM
T4 gp23 R	5'-TTGGGTGGAATGCTTCTTTAG-3'	10 uM

Media preparation. Bottom underlay LB agar was prepared with 1.5% agar, 1% tryptone, 0.5% yeast extract, 0.5% NaCl w/v. Top overlay agar was prepared with 0.75% agar, 1% tryptone, 0.5% yeast extract and 0.5% NaCl w/v, supplemented with 1mM $CaCl_2$.

T4 propagation. The T4 bacteriophage stock was prepared using a modified protocol from Marijn Celeen at Wageningen University. Overnight cultures of *E. coli* BL21 were prepared in LB medium at 37°C. The next day, 10 uL each of 1M $MgCl_2$ and 1M $CaCl_2$ were added to 10 mL of LB medium. The mixture was incubated at 37°C for 1 hour and 100 uL of high titer phage lysate (>10^8 PFU/mL) was added. After incubation at 37°C for 4 hours, chloroform was added when visible clearing was observed and the phage stock was stored at 4°C.

Quantification of T4 stock titer. T4 stock titer was quantified via double agar overlay plaque assays (13). Extracted T4 stock serial dilutions from 10^{-1} to 10^{-10} in LB media were prepared. An overnight culture of BL21 grown in LB broth at 37°C was diluted to 0.5 OD_{600}. 1 mL of culture was then combined with 100uL of a T4 dilution and allowed to incubate at 37°C for 8-12 minutes. The phage cell mixture was added to prewarmed 47°C overlay agar, vortexed, and poured on top of the solidified underlay agar plates and swirled briefly to ensure even spread. All plates were left on the bench to solidify at room temperature and then incubated overnight at 37°C. The T4 stock titer (PFU/mL) was determined based on plaque formation on countable dilution plates.

Qualitative assessment of capsule-mediated T4 resistance via double agar overlay plaque assays. Capsule-mediated T4 resistance was explored via double agar overlay plaque assays. The assay was conducted similar to the T4 quantification protocol. Overnight cultures for capsule producing E69, capsule knockout CWG655, T4 susceptible BL21, and T4 resistant DH5α were grown at 21°C and 37°C. E69 and CWG655 T4 negative controls and BL21 and DH5α 10^{-8} T4 dilution positive controls were plated and incubated at both temperatures. E69 and CWG655 testing conditions with 10^{-8} and 10^{-10} T4 dilutions were also plated in duplicates for both temperatures. The plates were qualitatively assessed for plaque formation.

RESULTS

PCR amplification and Sanger sequencing of *gp23* confirms T4 bacteriophage identity. To confirm the identity of our bacteriophage, PCR was used to amplify *gp23,* a

major capsid protein for T4 bacteriophage. The product was expected to be approximately 398bp, which was supported by imaging of the gel product (not pictured), along with sequencing of the amplicon. Upon entering the FASTA result from the sequencing reaction into NCBI Nucleotide Blast, it was shown that our query sequence matched that of T4 gp23 by 99%.

PCR amplification of wzy cassette and differential product sizes supports deletion of *wzy* gene cassette in CWG655. In order to confirm that *E. coli* strain CWG655, but not E69, had the *wzy* gene cassette deletion, a polymerase chain reaction was performed with EB6 and EB7 primers flanking the *wzy* gene cassette. A 4200 bp band was expected for E69 while for CWG655, a smaller band size was expected since the 1800 bp *wzy* cassette is replaced with a 944 bp kanamycin resistance cassette (14). The PCR gel showed a larger band size for E69 compared to CWG655. However, the band size for E69 is larger than 4200 bp which does not align entirely with the expected results obtained by Rana et. al (Figure 1).

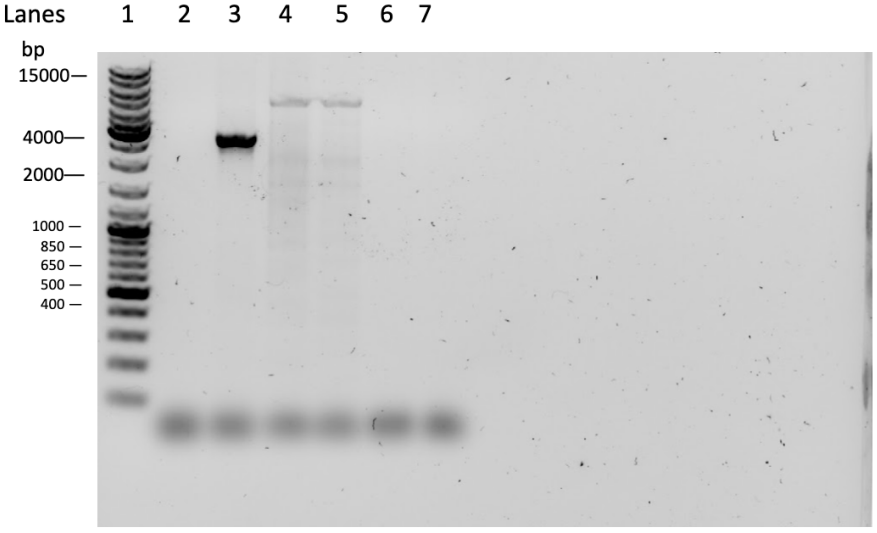

FIG. 1 PCR amplification of *wzy* cassette in *E. coli* strains E69 and CWG655. EB6 and EB7 primers were used to amplify the *wzy* cassette. The experiment was performed with two technical replicates for each strain. A 4200bp band was expected for E69, while a band <4200bp was expected for CWG655. Lane 1: 1Kb Pus DNA Ladder. Lane 2& 3: CWG655. Lane 4 & 5: E69. Lane 6 & 7: BL21 negative control *No product seen in lane 1.

T4 bacteriophage stock concentration was determined. In order to quantify our T4 stock concentration, a double agar overlay plaque assay was performed. T4 stock at varying dilutions and *E. coli* BL21 were mixed with overlay agar, poured over underlay LB agar plates, and incubated overnight at 37°C. The 10^{-8} dilution had countable plaques. The concentration of the phage stock was calculated to be 6.8 x 10^{8} PFUs/mL.

CWG655 was susceptible to T4 bacteriophage infection whereas E69 was resistant. To investigate the relationship between capsule production and T4 infection, we conducted a double agar overlay plaque assay to qualitatively assess possibility of capsule-mediated resistance. Bacterial strains BL21, DH5α, E69, and CWG655 were grown at 21°C and 37°C overnight. Cell cultures with 10^{-8} and 10^{-10} dilutions of T4 stock were mixed with overlay agar before pouring on underlay agar plates. Duplicates were performed for E69, and CWG655 testing conditions. Plates were then incubated overnight at their respective temperatures. Viral lysis was confirmed through the formation of plaques, and was observed only on CWG655 plates but not the E69 plates at 21°C (Figure 2A; 2B). The plaques were of the expected size for T4 (15), equally distributed, and lacked any abnormalities. The CWG655 (Figure 2E) and E69 (Figure 2C) phage negative controls exhibited fully grown bacterial lawns, indicating that both strains were growing. The T4 resistant DH5α exhibited a bacterial lawn and the T4 susceptible BL21 positive control plates were completely cleared as expected, confirming lytic activity (Figure 2D; 2F). These results suggest potential capsule-mediated T4 resistance in E69.

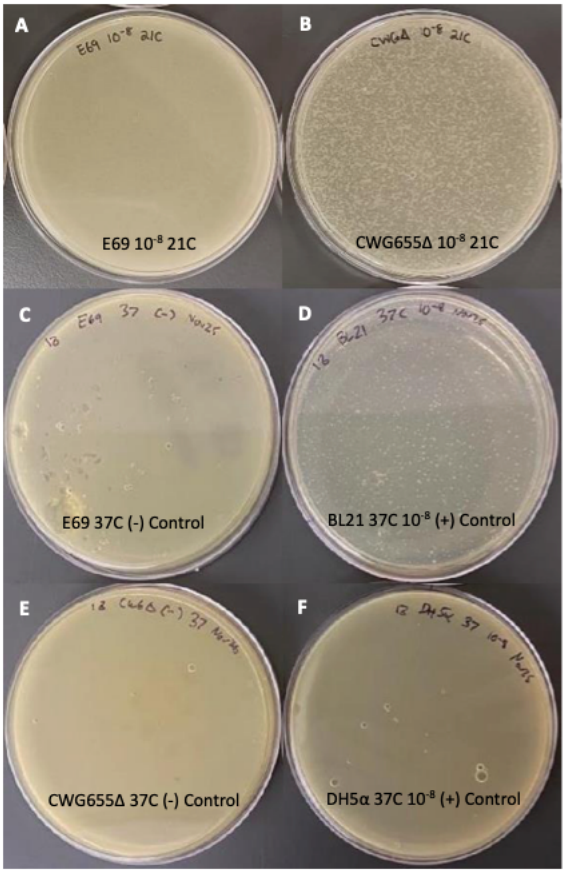

FIG. 2 Capsule knockout strain CWG655 was susceptible to T4 lysis whereas E69 was resistant. Overnight cultures of CWG655 and E69 grown at 21°C and 37°C were diluted to an OD_{600} of 0.5 before being subjected to a double agar overlay plaque assay at respective temperatures. (A) Capsule producing strain E69 and (B) capsule knockout strain CWG655 at 21°C. (C), (E) Negative controls were incubated without phage and displayed no viral plaque formation. Control *E. coli* strains (D) BL21 and (F) DH5α, known to be susceptible and resistant to T4 infection respectively, were incubated with T4 phage prior to assay.

DISCUSSION

Previous literature has emphasized the importance of bacterial capsules and their interactions with extracellular events, such as antibiotic resistance (11). Through this preliminary study, it was determined that chromosomal deletion of *wzy* in *E. coli* CWG655Δ may increase susceptibility to T4 bacteriophage infection compared to the wildtype strain, E69.

The identity of the bacteriophage was determined through PCR and Sanger sequencing of major capsid protein gp23, though there was sequence similarity to other phages such as enterobacteria phage T2, RB59, and RB55. The matches of our phage sample to these genomes can be explained through known similarities in capsid protein structures between phage families, as shown on the UniProt database when exploring proteins with 90% or greater amino acid sequence similarity to gp23.

Though we were able to confirm the identity of our bacteriophage, there were many difficulties faced in working with it. The procedures for attempted LB broth infectivity assays were problematic, likely contributing to unclear results; this was initially due to the lack of $CaCl_2$ and $MgCl_2$, which improve adsorption of T4 through cationic stabilization (16). This was corrected in subsequent assays by adding 0.001M of MgCl2 and CaCl2 based on a previous study (17). However, due to the limited volume of extracted T4 phage stock and its concentration, only an estimated MOI of 1 could be tested, and infectivity assays were carried out in test tubes to limit the number of bacterial cells present. The results were inconclusive, as the control strains did not behave as expected. Additionally, extreme care was taken when transferring T4, as aerosolization of the phage could contaminate other samples (18).

The PCR reactions conducted on our testing strains partially supported the deletion of the *wza-wzb-wzc* gene cluster in the knockout strain. Figure 1 shows a dark band at around 4000 bp in lane 2, which contains one of the 2 replicates of the knockout strain, whereas lanes 4 and 5, for E69, show a faint band higher than 4200 bp. There are no bands observed in lanes 6 and 7, which was BL21, a negative control. CWG655, the mutant strain, was created by electro-transforming *E. coli* CWG258 with plasmid WQ96, which was first generated by

cloning the region amplified by the EB6/EB7 primers into the BamHI-digested suicide vector pWQ173 and digesting the plasmid with BglII/BclI to remove an 1800 bp internal fragment. The digestion resulted in removal of all of the *wzb* genes and some of the *wza* and *wzc* genes (14). Then, gene replacement was allowed to take place via homologous recombination upon electroporation to generate CWG655. *E. coli* E69 is capable of capsule production, as it has intact *wza, wzb* and *wbc* genes, whereas CWG655 does not. This is supported by previous research showing restoration of capsule production in CWG655 after wzy gene complementation (14). The appearance of faint bands rather than dark bands in lanes 4 and 5 may have occurred because not enough cycles occurred during PCR, possibly due to PCR conditions not being optimal for *wzy* gene cassette amplification from the E69 template DNA. It is unlikely that insufficient DNA template was the issue, as E69 replicate #2 (lane 5) had the highest concentration of DNA (42.5 ng/uL) out of all the samples. Rather, suboptimal PCR conditions are a more plausible explanation for the faint bands. The PCR results by Rana et. al support this explanation since they obtained dark and distinct bands at around 4200 bp and 4000 bp by running their experiment with 35 PCR cycles compared to this study which contained only 25 cycles (12). Overall, these results do support differential capsule production between strains, as there is evidence of *wza-wzb-wzc* gene deletion in the CWG655 strain as shown by a smaller PCR product size, but the results could be further supplemented with additional tests. The PCR experiment needs to be optimized to conclude *wzy* gene cassette deletion with high certainty due to the presence of faint bands, primer dimers and discrepancies in the expected band sizes. Compared to the PCR results obtained by Rana et. al (12), the band sizes for E69 seemed larger than the expected 4200 bp. Previous studies have also used 1% DMSO to optimize PCR conditions (12). This could aid in prevention of non-specific amplification, faint bands and primer dimers seen at the bottom of the gel.

The preliminary experiment indicates that CWG655 is susceptible to T4 bacteriophage, possibly due to inhibition of capsule assembly. E69 was resistant to T4 infection, as both 21°C and 37°C conditions were completely devoid of plaques (Figure 2A). In contrast, CWG655 exhibited viral lysis across all phage dilutions at 21°C (Figure 2B). As 37°C is the optimal growth temperature for *E. coli*, we hypothesize that the higher growth rate may have resulted in different cell culture concentrations compared to 21°C. Since the same amount of virus was added to both temperature conditions, this difference would have caused different MOIs between the 21°C and 37°C conditions. We chose to include the 21°C test condition results in our figure as they showed the same result when replicated, whereas our 37°C plates were not consistent. It is possible that the CFU count in the CWG655 37°C trial overwhelmed the concentration of phage present, allowing more cells to survive, but this remains highly speculative and further testing is necessary to optimize the experiment. The CWG655 and E69 negative controls (Figure 2C; 2E), as well as our resistant DH5α positive control (Figure 2F) did not develop plaques, which was expected. Furthermore, the susceptible BL21 positive control showed no signs of bacterial growth (Figure 2D).

As mentioned in our introduction, the complicated energetics in the injection mechanism of the phage contributes to the difficulty in predicting resistance, and thus should further be explored. We propose one possible mechanism for resistance being that the presence of a capsule in the E69 strain physically prevents adequate lysosomal activity by the phage through blocking contact to the PG layer, and thus inadequate degradation, resulting in no genome injection (Figure 3A). This is supported by recent studies that suggest that T4 can adhere to the bacterial surface but is unable to lyse bacterial cells expressing K5 capsule (2). Alternatively, the presence of a capsule could impede or slow sheath contraction by acting as a resistor, which could disrupt the release of stored elastic potential energy leading to no rupture of the cell membrane and no phage DNA entering (Figure 3B). Finally, previous studies have shown that the capsule could act as a physical barrier by masking the receptors recognized by the tail fibers of T4 bacteriophage (Figure 3C), such as LPS and OmpC proteins found in the *E. coli* cell wall and cell membrane, respectively (10). Thus, capsule-mediated resistance towards T4 bacteriophage remains plausible.

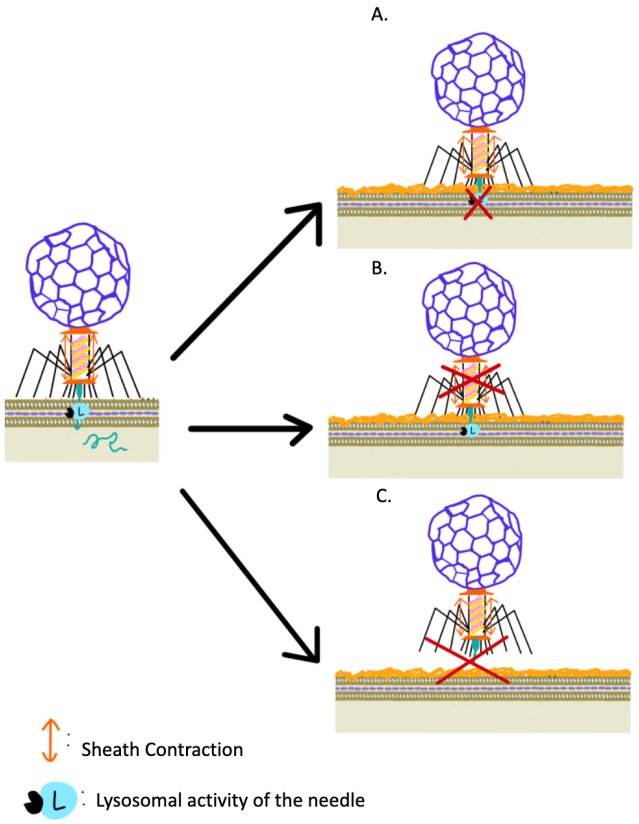

FIG. 3 Possible resistance mechanisms of *E. coli* E69 to T4 bacteriophage. (A) Presence of the capsule may prevent lysosomal activity of the phage needle. (B) Presence of the capsule may inhibit sheath contraction required for tail tip descent. (C) Capsule may act as a physical barrier by masking receptors bound by tail fibers.

Conclusions Previous studies have demonstrated the importance of bacterial capsules and their interactions with extracellular events. Here, we offer preliminary results suggesting that capsule expression confers T4 bacteriophage resistance. Through a qualitative assessment via double agar overlay plaque assays, it was observed that capsule producing strain *E. coli* E69 was resistant to T4-induced lysis and formed no viral plaques. CWG655 however, appeared to be susceptible and viral plaques could be seen across all T4 dilutions. We conclude that surface expression of CPS confers T4 bacteriophage resistance, but the mechanism remains unknown.

Future Directions To further confirm that the CWG655 strain contains a deletion, differential capsule production could be quantified using the phenol-sulfuric acid assay as performed by Botros et al. (1). However, there may be discrepancies as it quantifies all polysaccharides rather than the capsule specifically. Future experiments should also consider capsule staining to visually confirm capsule deletion and expression in the strains.

As our study relied on a single experiment, our preliminary finding of the interaction between T4 and capsulated E69 should be confirmed. Firstly, E69 resistance to T4 phage and CWG655 susceptibility should be confirmed through more conclusive infectivity tests. For one, an adsorption assay could be conducted to determine the adsorption rate of T4 in bacteria with and without capsule. As the lysis mechanism of T4 has been characterized by multiple phages adsorbing to a single cell and weakening the cell membrane (19), it would be beneficial to observe whether CPS positive and negative strains have similar adsorption rates. As this method of lysis can help release virions from T4-infected cells in liquid culture, repeating the liquid-broth infectivity assay would be beneficial.

Several alternative procedures such as spot testing or growth kinetics assays, could also be repeated to assess T4 infectivity (20). Although a preliminary spot test was attempted, no $MgCl_2$ or $CaCl_2$ was added to the LB plates, which may have inhibited phage adsorption and lead to unreliable results. A version of a growth kinetics assay in which OD_{600} of bacterial cells inoculated with varying concentrations of T4 was measured over a time period was attempted, but results were inconclusive. qPCR, flow-cytometry, and metabolic indicators could also be used to monitor bacterial growth (20).

One caveat to our experiment was issues with the maintenance of control strains. BL21 is susceptible to T4 infection, and DH5α is known to be resistant. Genome sequencing studies confirmed two CRISPR sequences present within the DH5α genome along with several prophages (21), and it is speculated that the two components play a role in the resistance to T4. During our experiments, there were several instances where DH5α cultures failed to grow in the presence of T4 while BL21 continued to grow, contradicting the literature. Therefore, performing a preliminary assay with a variety of strains that are susceptible or resistant based on the literature could allow for the selection of strains that give the most consistent results.

With any future experiments, a greater volume of T4 phage stock should be extracted before beginning experiments to allow replicates and a variety of MOIs to be tested. Higher MOIs would likely provide more definitive results and allow more options in terms of the starting OD_{600} and inoculation volume. This would allow the assay to provide qualitative (clearance of cultures) and quantitative (differential OD_{600} readings) results to confirm possible resistance or susceptibility against T4.

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CONTRIBUTIONS

All authors contributed equally to manuscript writing and editing. Protocol creation and experimental procedures were delegated and divided amongst team members. ES and JH lead efforts to propagate T4 and confirmed phage and bacterial strain identities via PCR, Sanger sequencing, and gel electrophoresis. AW and AC primarily focused on conducting the double agar plaque assays and quantifying T4 stock. All authors contributed to the assessment of experimental results as well as procedural troubleshooting.

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