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## **Development of a Microtiter Plate Assay for Real Time Analysis of T7 Bacteriophage Mediated Lysis of *Escherichia coli***

David Cho, Irene Lau, Michael Li, Dave Zhu

*Department of Microbiology & Immunology, University of British Columbia*

## **RcsB-Deficient *Escherichia coli* K-12 Do Not Exhibit Decreased Intrinsic Resistance Towards Antibiotics That Target the Cell Wall**

Paul McDade, Angela Wang, Vivian Wang, Clement Yau

*Department of Microbiology & Immunology, University of British Columbia*

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Irina Chan, Chi Wing Cheng, Jasmine Chin, and Veronica Chow

*Department of Microbiology and Immunology, University of British Columbia*

## Development of a Microtiter Plate Assay for Real Time Analysis of T7 Bacteriophage Mediated Lysis of *Escherichia coli*

David Cho, Irene Lau, Michael Li, Dave Zhu  
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When stressed, *Escherichia coli* releases soluble factors such as outer membrane vesicles. The release of these factors in the extracellular milieu has been shown to prevent bacteriophage infection in some cases. In this project, we developed an assay to study the effects of soluble factors on T7 bacteriophage infection of *Escherichia coli*. Our approach used a 96 well plate approach as an efficient alternative to plaque assays for monitoring phage infection of *Escherichia coli* based on optical density. Media taken from cultures of *Escherichia coli* mutants expected to show varied levels of outer membrane vesicle secretion were added to untreated wild-type *Escherichia coli* to test for resistance against T7 bacteriophage in a 96 well plate assay. Optical density decline of *Escherichia coli* treated with spent media from UB1005 and the four mutant strains were delayed compared the untreated *Escherichia coli* control. Taken together, these results implicate a protective effect due to the addition of spent media. We have successfully established a cost efficient and time-saving assay using a 96 well plate to track bacteriophage infection of bacterial cells.

Bacteriophage T7, a member of the Podoviridae family, replicates via a lytic life cycle within *Escherichia coli* (*E. coli*) [12]. T7 uses host lipopolysaccharide (LPS) as a receptor for adsorption and subsequent DNA insertion [8]. Phage adsorption specificity is mainly determined by the tail fiber protein and the structure of LPS on the host bacterial cell [12].

When placed under stressful conditions such as starvation, heat, cold, and increased osmotic pressure, bacterial cells achieve enhanced tolerance to other stresses in a process known as cross-protection [5]. Starvation-induced stress has been shown to provide cross-protection against antibiotics, chemical challenges, osmotic stress, and acid [5]. The mechanism of this response involves signal transduction systems that control expression of genes involved in stress response and cellular defense [5].

*E. coli* have previously been shown to have increased resistance to T7 bacteriophage adsorption following treatment with sub-lethal levels of kanamycin and gentamicin [3, 7, 9, 14]. It has been postulated that release of soluble factors into the culture supernatant mediates resistance to T7 bacteriophage [9]. *E. coli* have been shown to release surface LPS when stimulated with aminoglycoside antibiotics such as kanamycin and gentamicin [7]. However, a recent study showed that the addition of soluble LPS to *E. coli* did not reduce T7 bacteriophage infection [9]. Thus, the sole addition of LPS to *E. coli* (to simulate LPS release upon antibiotic treatment) is not sufficient to reduce T7 bacteriophage infection.

Outer membrane vesicles (OMV) have also been shown to be upregulated following addition of aminoglycoside antibiotics, and may play a role in bacterial defense [7, 11]. OMVs are released as a part of a regulated stress response [16]. *E. coli* mutants that overproduce OMVs show increased survival against 10% ethanol and polymyxin B [16]. These OMVs are made of outer membrane and periplasmic material, and are released without impacting membrane integrity of the cell [16].

A previous study showed that an OMV-overproducing *E. coli* mutant survived treatment of antimicrobial peptides better than a wild-type (WT) strain [14]. When treated with low concentrations of OMVs, *E. coli* cultures show increased survival and development of resistance against polymyxin B [14]. Moreover, co-incubation of T4 bacteriophage with purified OMVs results in irreversible binding and significantly reduced T4 infection [14]. Thus, we propose that soluble factors, such as OMVs, are a key factor also involved in increased resistance against T7 bacteriophage infection.

To investigate the effects of OMVs on T7 bacteriophage infectivity to *E. coli*, we used mutants known to overproduce and underproduce OMVs [11]. OMVs released into the media contain LPS on the surface which acts as a decoy receptor for T7 infection as shown in Figure 1 [9, 17]. T7 DNA ejection into OMVs effectively decrease the number of viable phages present thus reducing bacterial cell infection.

To this end, we developed a high throughput assay to track changes in optical density over time in response to phage infection. To our knowledge, no high throughput assay exists for this specific purpose. In this study, we describe

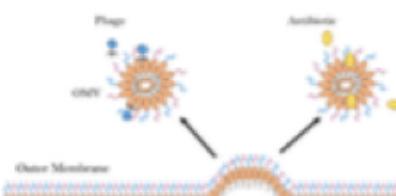


FIG. 1 Illustration of OMVs acting as decoys to protect the bacterial cell from threats such as phage and antibiotics. Adapted from: Schneichleiner C, Kuhn MJ. 2015. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. Nat Rev Microbiol. 13:605-619. [17].

this novel assay to measure phage infection thereby bypassing the long incubation periods and the need for counting plaques as required in previous phage assays [1, 15, 18].

### MATERIALS AND METHODS

Strains used in this study. *E. coli* UB1005 was obtained from the Hancock Laboratory and plated on lysogeny broth (LB) media. *E. coli* strains *Δ*agP JW0157, *Δ*agP JW5100, *Δ*agP JW3841, and *Δ*cpk JW1221 were obtained from the Keio Collection and plated on LB media with kanamycin at a concentration of 50μg/mL. *Δ*agP JW0157 and *Δ*agP JW5100 are hypersecretion *E. coli* strains. *Δ*agP JW3841 is an OMV underproducing *E. coli* strain. *Δ*cpk JW1221 is used as a medium deletion control that is not known to have any effect on OMV production [11]. Bacteriophage T7 was provided by the Department of Microbiology and Immunology at UBC.

**M9 minimal media.** 1L of M9 minimal media was created with 100mL 10X M9 salts, 50mL 10% w/v glucose, 10mL 0.1M MgSO<sub>4</sub>, 50mL 20% w/v casamino acids, 2mL filter sterilized 10mg/mL vitamin B1, 2mL filter sterilized 10mg/mL tryptophan, and 750mL deionized water. 10X M9 salts were composed of 60g Na<sub>2</sub>HPO<sub>4</sub>, 30g K<sub>2</sub>HPO<sub>4</sub>, 5g NaCl, 10g NH<sub>4</sub>Cl, and 999mL distilled water. 1mL 1M filter sterilized CaCl<sub>2</sub> was added to the 10X M9 salts mixture following an autoclave cycle. All components of the media were sterilized separately, either through an autoclave cycle or through filter sterilization, and compiled together under aseptic conditions.

**Isolating strains.** For the purpose of obtaining isolated colonies, UB1005 was streaked onto 1.5% LB agar plates. *Δ*agP, *Δ*cpk, *Δ*agP, and *Δ*cpk mutants were received on filter disks and were subsequently placed on 1.5% Lysogeny Broth (LB) agar plates supplemented with 50μg/mL kanamycin salt from Invitrogen™. Plates were incubated overnight at 37°C and stored at 4°C for future use.

**DNA isolation of *E. coli* mutants.** Using the isolated colonies from LB-Kan plates, overnight liquid cultures of *Δ*agP, *Δ*cpk, *Δ*agP, and *Δ*cpk mutants in LB containing 50μg/mL kanamycin were prepared for DNA isolation. 2 × 10<sup>8</sup> cells from each overnight culture were used for genomic DNA isolation with a PureLink® Genomic DNA Kit from Invitrogen™. The amount of cells was calculated using 1 OD<sub>600</sub> = 8 × 10<sup>8</sup> cells/mL. The DNA concentrations and absorbance spectra were measured using the Nanodrop spectrophotometer. These samples were subsequently used for PCR amplification of the kanamycin cassette in each mutant.

**Oligonucleotide primer design.** The polymerase chain reaction (PCR) primers for the mutant strains were individually designed to amplify the kanamycin cassettes (Table 1). The primers were designed to be complementary to upstream and downstream regions approximately 200 base pairs from the respective genes that were knocked out and replaced with a kanamycin cassette on the parent strain *E. coli* BW25113. The forward and reverse primers were designed using software provided by Integrated DNA Technologies.

**PCR and agarose gel electrophoresis.** PCR amplification of the DNA samples from *Δ*agP, *Δ*cpk, *Δ*agP, and *Δ*cpk mutants was carried out according to the Platinum™ Taq DNA Polymerase protocol from Invitrogen™. 1.5μL of 10X loading dye per 15 μL of PCR product and 5μL of TrakH™ 1KB Plus DNA Ladder by Invitrogen™ were used for gel electrophoresis. These samples were run on a 1% agarose gel for 1.5 hours at 110 V. PCR products were purified using the PureLink® PCR Purification Kit from Invitrogen™ in preparation for Sanger sequencing.

TABLE 1 Forward and reverse primer sequences used to amplify the kanamycin cassettes on the mutant strains.

Description	Sequence (5'→3')	T <sub>m</sub> (°C)	% GC Content
UB1005	GCGCTTAATTCGCGGAAAT	55.0	49%
ΔagP	GGCAACTTGTTGCGGAGAAG	55.0	49%
Δcpk	CTTAACTGGTTGTTGCGGAA	55.7	49%
ΔagP	TTGCGGAAATGCGGAAATGCG	55.8	49%
Δcpk	CTTAACTGGTTGTTGCGGAA	55.9	49%
Δcpk	ACGAAAGGAAATGCGGAAATGCG	55.7	49%
Δcpk	ATATTAATGCGGAAATGCG	55.9	49%
Δcpk	GGCGAAATGCGGAAATGCG	55.0	49%

**Sanger sequencing preparation.** The concentration of the purified PCR products were measured using nanodrop. These samples were diluted to a concentration of 15ng/μL in a total volume of 10μL. 10μM forward (F) and reverse (R) primers were prepared in separate tubes for each sample. Primers were diluted to 5pmol/L in a total volume of 10μL. The PCR products, along with the respective primers, were sent to the University of British Columbia's sequencing unit (NAPS) in the Michael Smith Laboratories.

**Growth curves.** 30 mL of M9 minimal media was added to five sterile 250 mL Erlenmeyer flasks, one for each strain. Three isolated colonies from each strain were inoculated in their respective flasks, which were subsequently placed on a shaker at 37°C. The optical density at 600nm of each culture was measured in 30 minute intervals.

**Isolating spent media from overnight cultures.** For the purpose of isolating spent media, strains were propagated on a shaker overnight in liquid M9 minimal media at 37°C. For certain experiments, UB1005 was also cultured in liquid LB. For all experiments, 20mg/mL glutamine was added to the *Δ*cpk culture as GlnA is a glutamine synthetase and thus the knockout strain could not synthesize glutamine independently. 20mg/mL glutamine was also added to the culture media of all other strains to control for glutamine supplementation. For one experiment overnight cultures were distributed into Eppendorf Tubes® and centrifuged at 5000 × g for 1 minute. The supernatant was transferred into a single 15mL Falcon Tube™. Remaining pellets were re-suspended and collected in 1mL of warm M9 minimal media and spun once more at 5000 × g for 1 minute. The supernatant was transferred into the Falcon Tube™ and the pellet was discarded. The collected spent media was then stored at 4°C for future use.

**96 well plate phage infectivity assay.** For the purpose of obtaining viable cells in growth phase, UB1005 was cultured on a shaker in liquid M9 minimal media at 37°C for no more than 4 hours. The optical density at 600nm (OD<sub>600</sub>) was measured on a spectrophotometer to determine the number of cells present. The amount of T7 bacteriophage required for the desired multiplicity of infection was calculated using the following equation:

$$1 \text{ OD}_{600} = 8 \times 10^8 \text{ cells/mL}$$

MOI = phage

The T7 bacteriophage stock was diluted using M9 minimal media. The UB1005 culture was distributed into Eppendorf Tubes® and spun at 5000 × g for 1 minute. The supernatant was discarded and the cell pellets were re-suspended in warm spent media. 270μL of UB1005 re-suspended in spent media and 30μL

of diluted T7 bacteriophage was added to each well in a 96 well polystyrene plate. A replicate number of 5 was used for each spent media condition. The controls were added (as per below) to the plate and the absorbance at 600nm in each well containing samples was measured on an Epoch Microplate Reader in 10 minute intervals. The plate was incubated at 37°C between each read.

**Controls for the 96 well plate assay.** 30  $\mu$ L of diluted T7 bacteriophage was added to 270  $\mu$ L of the UB1005 culture in growth phase in a single well to control for the effects of spent media from overnight cultures. 300  $\mu$ L of the UB1005 culture in growth phase was added to a single well to show the same effect. 300  $\mu$ L of M9 minimal media and LB were added to single wells and were used as blanks for the plate reader.

## RESULTS

**Confirming the identity of the *E. coli* mutants.** To verify the identity of our mutants, we performed PCR and Sanger sequencing. The bands shown in Figure 2 from all four of the mutant strains were approximately 1.6 kilobase pairs. The DNA sequencing results were aligned to the template vector pKD13 with 73–74% query and 99% identity, and an E value of 0. The primer sequences flanking the kanamycin cassette provided by the Celi Genetic Stock Center website used for creating the knockouts were matched with the fasta sequences of the PCR products [<https://gsc2.biology.yale.edu/KeioList.php>] (Table S1A and S1B). Through this, we have confirmed the identity of the *E. coli* mutants.

**Standard growth curves of all strains were established.** The growth curves in Figure S1 show that UB1005,  $\Delta$ ycfL, and  $\Delta$ degP reached exponential phase at about 180 minutes post inoculation, whereas  $\Delta$ glbA and  $\Delta$ olB reached exponential phase at about 300 minutes. UB1005 entered stationary phase earlier than  $\Delta$ ycfL and  $\Delta$ degP, although the growth of these strains also began to

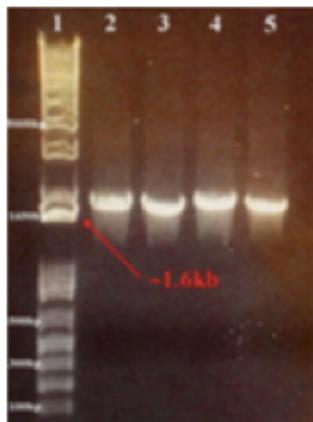


FIG. 2 1% agarose gel containing PCR products of the amplified kanamycin cassette run at 130V for 1.5 hours. Lane 1, 1Kb TrackIt Plus DNA Ladder. Lane 2,  $\Delta$ degP. Lane 3,  $\Delta$ glbA. Lane 4,  $\Delta$ ycfL. Lane 5,  $\Delta$ olB.

slow down towards the last few timepoints. Through this, we have established and visualized the growth curves of all strains.

### T7 bacteriophage infection occurs in 96 well plate assay

**assay.** To determine whether T7 bacteriophage infection in exponential growth phase *E. coli* UB1005 can occur in a 96 well plate, UB1005 cell culture and T7 bacteriophage solutions were added at a 9:1 volume ratio at MOIs of 0, 0.5, 1, 5, and 10 in replicates of 5 (Fig. 3A). A growth curve analysis of UB1005 inoculated with T7 at MOIs of 0 and 10 in a more conventional method utilizing Erlenmeyer flasks was conducted as a control (Fig. 3B). This curve was monitored concurrently with the plate assay for comparison and to serve as a positive control for the effects of T7 in the scaling down of the microtiter plate assay. In the plate assay, all sample condition wells exhibited an initial OD<sub>600</sub> of 0.3 at the time of virus inoculation. The OD<sub>600</sub> values in sample condition wells at MOIs of 0.5, 1, 5, and 10 began to decline 45 minutes post-inoculation and stabilized below 0.1 OD<sub>600</sub> approximately 24 hours post-inoculation (data not shown). In contrast, sample condition wells at MOI of 0 showed continued growth 4.5 hours post-inoculation, reaching 0.89 OD<sub>600</sub> by the conclusion of the assay (data not shown). The growth curve analysis at MOI of 10 exhibited similar trend to sample condition wells at MOI of 10. The growth curve

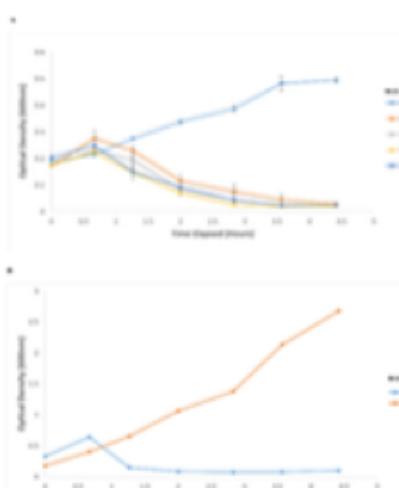


FIG. 3 A) Multiplicity of infection on growth of *E. coli* UB1005 in 96 well plate assay. Standard deviation shown as error bars. n = 5. UB1005 was cultured on a shaker in liquid M9 minimal media at 37°C until an optical density greater than 0.3 was reached. UB1005 was resuspended in spent media and inoculated with T7 bacteriophage at an MOI of 0.03. OD<sub>600</sub> readings were taken every 10 minutes. The negative control showed continuous growth of culture, whereas all other wells with T7 bacteriophage demonstrated a decline in optical density measurements towards the end of the experiment. B) Optical densities of each strain at 150 minutes and 200 minutes.

analysis at MOI of 0 had a much higher growth rate in comparison to sample condition wells at MOI of 0, reaching 4.9 OD<sub>600</sub> at the conclusion of the assay (data not shown). Through this, we show that T7 bacteriophage infection occurs in the microtiter plate assay.

### Cells treated with spent media show a delayed decline in optical density in T7 bacteriophage infection.

To determine whether spent media confers protection against T7 bacteriophage infectivity, a 96 well plate assay was conducted at a MOI of 0.03 with spent media from overnight cultures of all strains in M9 supplemented with 20mg/mL of glutamine (M9+Gln) (Fig. 4A and 4B). UB1005 was also cultured in LB and un-supplemented M9. Untreated conditions with and without T7 inoculation served as positive and negative controls, respectively. OD<sub>600</sub> values in all spent media conditions began to decline at a later time in comparison to the positive control. Spent media from UB1005 in LB and UB1005 in M9+Gln led to a decrease in OD<sub>600</sub> values 40 minutes earlier in comparison

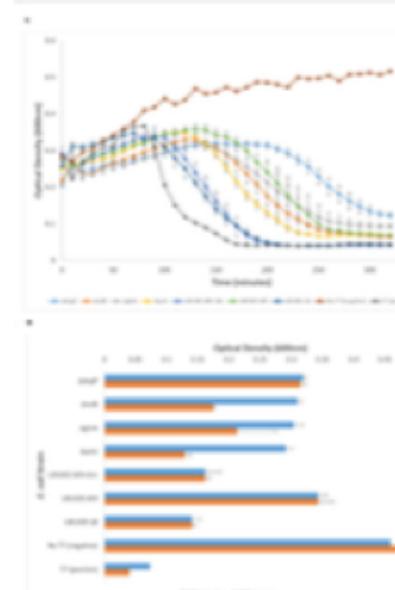


FIG. 4 96 well plate assay. Standard deviation shown as error bars. n = 5. UB1005 was cultured on a shaker in liquid M9 minimal media until an optical density greater than 0.3 was reached. UB1005 was resuspended in spent media and inoculated with T7 bacteriophage at an MOI of 0.03. OD<sub>600</sub> readings were taken every 10 minutes. The negative control showed continuous growth of culture, whereas all other wells with T7 bacteriophage demonstrated a decline in optical density measurements towards the end of the experiment. B) Optical densities of each strain at 150 minutes and 200 minutes.

to spent media from UB1005 in M9. Spent media from  $\Delta$ glbA,  $\Delta$ ycfL, and  $\Delta$ degP in M9+Gln showed comparable growth dynamics to spent media from UB1005 in M9, with OD<sub>600</sub> values beginning to decline at 130 minutes, 120 minutes, and 140 minutes post-inoculation, respectively. Spent media from  $\Delta$ degP in M9+Gln led to the most delayed decline in OD<sub>600</sub>, initiating at 190 minutes. Through this, we show that a delayed decline in optical density occurs in cells treated with spent media and inoculated with T7 bacteriophage.

## DISCUSSION

The purpose of this study was to investigate the effects of spent media on modulating T7 bacteriophage infection in *E. coli* cells. Previous studies have shown that LPS and OMVs are separately able to prevent T7 infection in *E. coli* [3, 11, 14]. Using OMV overproducing mutants JW0157 ( $\Delta$ degP) and JW5100 ( $\Delta$ olB), OMV underproducing mutant JW3841 ( $\Delta$ glbA), and a random mutant not affecting OMV production JW1221 ( $\Delta$ ycfL), we were not able to determine definitively whether or not OMVs are a crucial part of inducing resistance against T7 bacteriophage infection. The effects on OMV production were predicted phenotypes. As we did not characterize the spent media for any factors that may be present, it cannot be stated with any certainty whether or not the corresponding phenotypes manifested. However, through our 96 well plate assay, we did specifically observe an interesting trend in which the transfer of spent media from all 4 mutant strains in M9+Gln and UB1005 in un-supplemented M9 minimal media resulted in delayed cell lysis compared to spent media from UB1005 in LB and M9+Gln.

We describe a novel 96 well plate assay to track changes in bacterial cell culture turbidity over time, measured in optical density at 600 nm. Briefly, *E. coli* UB1005 is grown to an optical density of 0.3 in Erlenmeyer flasks. The cells are isolated and re-suspended in previously isolated spent media before seeded into 96 well plates. Following this, T7 bacteriophage is added to the wells and the initial time point is taken. We show that *E. coli* is capable of growing in 96 well polystyrene plates with uniform growth rates across replicate wells (Figs. 3A, 4A, and 4B). We also show that addition of T7 bacteriophage to *E. coli* in exponential growth phase leads to a decrease in OD<sub>600</sub> values over time in 96 well plates, producing similar growth dynamics to a more conventional growth assay setup in an Erlenmeyer flask under the same MOI (Fig. 3A and 3B). These results suggest T7 bacteriophage infection as the cause for decrease in cell culture turbidity, substantiating the plate assay as a robust, high throughput method to investigate interactions between phage and bacteria cells.

Previous phage assays relied on visual enumeration of plaques [1, 15, 18]. Disadvantages with these systems

include the use of a semi-solid overlay, long incubation times across multiple days for plaques visibility, and manual plaque counting. Our 96 well plate assay eliminates these disadvantages by using a plate reader to measure optical density readings over time. As phage solutions are added directly into the wells containing bacterial cultures, a semi-solid overlay is not required. Incubation times are dependent on the MOI selected for the experiment. In general, 12 hours is sufficient for near complete cell lysis and stable optical density readings. Through this assay, it is possible to track phage infection over time, as optical density readings decrease over time with increased phage infection and bacterial cell lysis. This eliminates the need for manual counting of phage plaques. Certain drawbacks to take into consideration for future experiments include evaporation, which can be partially controlled by doing measurements then quickly covering the plate with a lid. However, long experiments are more prone to errors caused by evaporation.

We first tested a range of MOIs from 0 to 10 and observed that all non-zero MOIs tested resulted in nearly identical death rates (Fig. 3A). However, any components in the spent media that can neutralize T7 bacteriophage may be overwhelmed by a higher MOI. Thus, the chosen MOI of 0.03 would likely still effectively infect and lyse all cells within a reasonable time, and not high enough so that the virus is overwhelming.

Despite the difference in growth rates amongst all five *E. coli* strains, the exponential phases for these strains started at approximately 0.3 OD<sub>600</sub>. The exponential phase is a period of time in which the *E. coli* cell population rapidly increases along with the production of growth-essential machinery that could be exploited by T7 bacteriophage. This growth phase is ideal for T7 bacteriophage infection because *E. coli* cells in stationary phase have been shown to be more immune to infection [4].

Employing our 96 well plate assay, we demonstrate that re-suspension of exponential growth phase UB1005 in spent media confers a protective effect against T7 bacteriophage infection. This manifests in the form of delayed decrease in OD<sub>600</sub> values in all spent media conditions compared to control cells not treated with spent media (Fig. 4A and 4B). We had anticipated that soluble factors released by the various knockout strains and UB1005 would lead to a mode of resistance against phage infection and the results provide support for this notion. It is worth noting that cells treated with spent media from UB1005 in M9+Gln or UB1005 in LB began lysing on a noteworthy scale earlier than cells treated with spent media from UB1005 in M9. A possible explanation for this observed difference is that UB1005 spent culture media composed of M9+Gln or LB may not stimulate sufficient stress to induce a stringent response in UB1005. However, the growth dynamics of cells

treated with spent media from UB1005 in M9 was similar to cells treated with spent media from the four mutant strains. Therefore, OMVs may instead play a more minor role in T7 bacteriophage resistance than previously anticipated [14]. Spent media from *Agy57* (unrelated OMV production) and *Agy64* (OMV underproduction) mutants do not result in earlier optical density decline in comparison with spent media from OMV overproducing mutants. This implicates the presence of other soluble factors apart from OMVs that may facilitate resistance to T7 bacteriophage infection. A plausible explanation for this observation and the failure to replicate the result shown by Manning et al. is that OMV composition can be distinct and variable amongst different species and growth environments [17]. Manning et al. cultured enterotoxigenic *E. coli* in LB, while we cultured *E. coli* UB1005 in M9. The differences point to a possibility that the OMVs produced from UB1005 were not composed of molecules favouring T7 adsorption, and were therefore unsuitable to act as decoys. Furthermore, the observed protective effects could also be attributed to intrinsic changes within the cell, such as the induction of the RpoS pathway due to nutrient starvation, as opposed to attributing the effects to released soluble factors [5].

Herein, we describe a novel 96 well plate assay for quantifying T7 bacteriophage infection over time in *E. coli* cells. Using *E. coli* UB1005 and T7 bacteriophage, we tested a range of MOIs and measured optical density at 600 nm over the course of T7 infection. We show that our assay is functional across an MOI range of 0 to 10. The 96 well plate phage assay also shows a similarly shaped curve of optical density over time compared to growth of *E. coli* in a 50 mL flask at a tested MOI of 10. Using spent media isolated from OMV over- and underproducing mutants, and UB1005 grown in different media conditions, we observed a trend that cells treated with spent media from UB1005 in M9+Gln or UB1005 in LB began lysing earlier than cells treated with spent media from UB1005 or mutant strains in M9. Due to differing growth conditions, we discussed variable OMV composition as a possible factor influencing the observed results. However, we were unable to definitively determine the role of OMVs in T7 bacteriophage resistance in *E. coli* UB1005. Lastly, the development of the 96 well plate phage assay will allow more efficient tracking and quantification of bacteriophage infection courses. This assay may be further utilized for other bacteriophages and bacterial species.

#### FUTURE DIRECTIONS

To further investigate the extent of OMV involvement in protection against T7 bacteriophage infection, it may be useful to experiment with supplementing *E. coli* cultures with purified OMVs in the 96 well assay. The addition of purified OMVs would avoid extraneous effects from any

unnecessary contaminants that may be present in spent media that may affect results. As the previous study on T7 bacteriophages suggests, we expect to elucidate a more definitive relationship between OMVs and resistance to T7 bacteriophage infections following the addition of purified OMVs [3].

Another direction of investigation may be quantifying the OMV content in the spent media of OMV overproducing and underproducing mutants. This may provide more concrete numerical correlations between OMV levels and T7 bacteriophage infectivity. Using transmission electron microscopy (TEM), quantification of membrane vesicles can be performed [2]. The outer membrane vesicles used during TEM can be isolated and collected using the density-gradient centrifugation method [13]. Alternative methods may include the usage of scanning ion occlusion sensing (SIOS) for a more rapid quantification process which eliminates the need for isolating OMVs [6].

Furthermore, characterization and identification of specific soluble factors released by UB1005 under stringent and non-stringent conditions may provide information on other soluble factors that also contribute to protection against T7 bacteriophage infection. Comparative whole genome transcriptome analysis results would also allow for the identification of candidate released factors that may be involved in T7 bacteriophage resistance. Examples of stringent conditions may include the addition of aminoglycoside antibiotics such as kanamycin and gentamicin or heat [10]. Non-stringent conditions may be achieved by growing UB1005 in lysogeny broth.

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