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Antisense RNA Knockdown and Genomic Deletion of *wecD* Sensitizes *Escherichia coli* to Bacteriophage T7 Infection

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WecD is an essential enzyme in the biosynthesis of enterobacterial common antigen (ECA), an outer membrane glycophospholipid that is conserved among all members of the Enterobacteriaceae family, including *Escherichia coli*. Mutations of *wecD* disrupt ECA biosynthesis and result in a hypervesiculation phenotype characterized by increased production of outer membrane vesicles (OMVs). OMVs have been shown to occur during the onset of bacteriophage adhesions to host cells, which is a proposed mechanism of bacterial avoidance of phage infections. In this study, we investigated whether silencing WecD expression increased resistance to bacteriophage T7 in *E. coli* by (1) silencing *wecD* expression using antisense technology and (2) genomic deletion of *wecD* from the Keio strain collection. Counter to our hypothesis, both *wecD* asRNA knockdown and *wecD* genomic deletion appeared to sensitize *E. coli* to bacteriophage T7 infection. Additionally, and in line with our expectation that genomic deletion is a more effective means of mitigating gene expression as compared to using an asRNA knockdown, *wecD* genomic deletion was observed to increase *E. coli* sensitivity to bacteriophage T7 infection to a greater extent than *wecD* asRNA knockdown. Based on our observations, we propose that the presence of antibiotics and the silencing of *wecD* may induce cellular and membrane stress in the OM and compromise the physical protection of the mutant cells to bacteriophage T7 infection, therefore contributing to this increased sensitization to phage infections observed in *wecD*-silenced cells.

Bacteriophage are viral entities that infect and replicate within bacterial cells (1). Given the rising incidence of antibiotic-resistant bacteria, phage use has been proposed as an alternative therapeutic for treating severe bacterial infections (2). However, before phage therapy can be adopted as a standard treatment in current clinical practices, it must overcome scientific, financial, and regulatory hurdles (2). Notably, phage resistance in bacteria can be a major impediment to the efficacy and advancement of phage therapy (3).

Enterobacterial common antigen (ECA) is a glycophospholipid embedded in the outer leaflet of the outer membrane (OM) in Gram-negative enteric bacteria, including *Escherichia coli* (4). Although the function of ECA is poorly characterized, it is conserved among all members of the Enterobacteriaceae family and therefore a target for immunotherapy against antibiotic-resistant bacteria (4). The Wec proteins are involved in ECA biosynthesis, a process which requires the addition of three modified sugars onto a universal lipid carrier in the inner membrane (IM) before being translocated to the outer leaflet of the OM (5). WecD, a TDP-fucosamine acetyltransferase, is known to be a key enzyme in the biosynthesis of ECA (5). Specifically, WecD catalyzes the acetylation of the final sugar added to the end of the ECA sugar chain(5).

In a previous study, McMahon *et al.* found that a stress response in the OM or periplasm, induced by the blockage of ECA biosynthesis, results in a hypervesiculation phenotype (6). Gram-negative bacteria, including *E. coli,* are known to produce outer membrane vesicles (OMVs) as a stress response during which the vesicles play many key roles in bacterial survival including nutrient acquisition, biofilm development, and pathogenesis (7). More importantly, vesiculation is observed to increase during the onset of phage adhesion and has been proposed as a mechanism which helps bacteria avoid infection by immediately releasing the phage before the phage DNA is injected and by creating an abundance of decoy membranes in the environment (7, 8). In agreement with this hypothesis, Hardman *et al.* suggested that OMV production may be responsible for the observed decreased phage infectivity of their antibiotic-treated *E. coli* cells (9).

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Other related studies have also shown that a stress response, induced by antibiotic treatments, results in *E. coli* cells being less susceptible to phage infections (10, 11). Based on these results, it is therefore plausible that the hypervesiculation phenotype observed from the disruption of ECA biosynthesis may also lead to phage resistance in *E. coli*.

In a study conducted by McMahon *et al.*, *wec* mutations in the ECA synthesis pathway resulted in a hypervesiculation phenotype in Gram-negative *Serratia marcescens* (6). Insertion mutations in *wecA* (using pknock-Cm), *wecD* (using mini-Tn5 Km), and *wecG* (using pknock-Cm) were observed to result in the accumulation of undecaprenol monophosphate, lipid I, or lipid II, respectively, which are intermediate molecules of ECA (6). Importantly, McMahon *et al.* suggested that this accumulation of ECA intermediates was due to the loss of one or all forms of ECA inducing hypervesiculation because despite the accumulation of different lipid intermediates, all the mutations have upregulated vesiculation levels (6). Furthermore, they observed that *wecD* mutations produced an approximately 20-fold increase in vesiculation in comparison with the wild-type (WT), which was a higher increase than those observed following mutations of any of the other Wec proteins (6).

To investigate whether silencing *wecD* expression increased resistance to phage infection in *E. coli*, we set out to construct a plasmid to silence *wecD*. Specifically, the plasmid we designed contains an antisense RNA (asRNA) component; when this component is expressed, it silences the target gene (12). In our experiment, the asRNA transcript, expressed from the plasmid, is designed to complement to the ribosomal binding site (RBS) sequence in *wecD* mRNA and silence the gene in the host cell by hybridizing to the *wecD* mRNA to thereby prevent ribosome binding and subsequent *wecD* translation.

We chose to incorporate the asRNA sequence into plasmid pHN678 since this plasmid possesses features that allow optimal antisense function. Firstly, pHN678 contains a *lac* operator sequence that allows for isopropyl β-D-1-thiogalactopyranoside (IPTG) induction control of the asRNA sequence (13). In addition, when expressed, the pHN678 transcript forms a paired termini secondary structure flanking the asRNA, which stabilizes the asRNA molecule and increases its resistance to degradation by dsRNA-specific RNAses (13). An increased stability allows for accumulation of the asRNA when pHN678 is induced with IPTG, which eventually helps amplify the silencing of *wecD* (13).

To study the effect of *wecD* on *E. coli* resistance to bacteriophage T7, we compared the cellular growth rate and phage resistance level of the WT strain to the *wecD* genomic deletion strain and the *wecD* asRNA knockdown strain. We hypothesized that both the *wecD* genomic deletion and *wecD* asRNA knockdown strains would result in hypervesiculation and subsequently reduce bacteriophage T7 infectivity in both strains. However, our data has shown the opposite effect. Both the genomic deletion and asRNA knockdown of *wecD* appear to sensitize *E. coli* to bacteriophage T7 infections, and the effect in both experimental model systems is very similar.

MATERIALS AND METHODS

Strains and plasmids used. *E. coli* K-12 substrain BW25113, MG1655, and DH5α cells harboring pHN678 and pZ plasmids were obtained from the Ramey culture collection (Department of Microbiology and Immunology, University of British Columbia). *E. coli* K-12 substrain JW5597-1 was obtained from the *E. coli* Genetic Stock Centre (CGSC). The *E. coli* strains and plasmids used in this experiment are summarized in Table 1. MG1655 was used to harbor pHN678, pZ, or the constructed pKAT*wecD* plasmid which contains the *wecD* antisense insert. As a comparative model, BW25113 was used as the parent/WT strain for the *wecD* genomic deletion JW5597-1 strain.

Growth conditions of the strains. All strains were grown at 37°C on lysogeny broth (LB) plates (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, 1.5% w/v agar, pH 7.5) with 30 µg/mL chloramphenicol to select for cells containing pHN678,

TABLE 1 Summary of *E. coli* strains and cloning vectors used.

***E. coli* K-12 Strain**	**Description**	**Genotype**
MG1655	Wild type control; Host strain for pHN678 and pKAT*wecD*	F-, *λ-*, *rph-1*
BW25113	Wild type control for JW5597-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ-*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*
JW5597-1	Harbors a deletion in *wecD*	*F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1, ΔrffC741::kan, Δ(rhaD-rhaB)568, hsdR514*
Cloning Vectors		**Features**
pHN678	Cloning vector used for making the pZ and pKAT*wecD* construct	IPTG-inducible promoter [*Ptrc*], paired-termini flanking MCS, Cam^R
pZ	Knockdown *ftsZ* expression upon IPTG induction which results in observable cellular elongation	pHN678 derivative, IPTG-inducible promoter [*Ptrc*], paired-termini flanking MCS, Cam^R, *ftsZ* antisense insert
pKAT*wecD*	Knockdown *wecD* expression upon IPTG induction	pHN678 derivative, IPTG-inducible promoter, paired-termini flanking MCS, Cam^R, *wecD* antisense insert

pZ, or pKAT*wecD*, and with 50 µg/mL kanamycin for the JW5597-1 strain. Overnight cultures were grown in LB (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, pH 7.5) with the same concentration of antibiotics for selection, at 37 °C and shaken at 200 rpm for 18 hours.

Generation of MG1655 competent cells. The Hancock Lab's protocol for calcium chloride ($CaCl_2$) transformation of *E. coli* was used with some modifications (14). From overnight *E. coli* MG1655 culture, fresh LB broth was inoculated (1:100 dilution) and grown at 37 °C on a shaker until OD_{550} of 0.2 to 0.4 was achieved. The cells were then incubated on ice for 10 minutes, pelleted at 4 °C and 10,000 rpm for 5 minutes in a Beckman Coulter JA-20 Fixed Angle rotor, resuspended in half volume of 0.1 M $CaCl_2$, incubated on ice for 30 minutes, and then centrifuged again. After resuspending in 1:50 of the original culture volume in 0.1 M $CaCl_2$ and incubating on ice for an hour, an aliquot of the cell suspension was mixed with sterile 25% glycerol (1:1) and stored frozen at -80 °C until use.

Antisense insert sequence design. The *wecD* sequence was determined from the complete genome of *E. coli* K-12 substrain MG1655 (accession number: NC_000913.3), available in the GenBank database (https://www.ncbi.nlm.nih.gov/nuccore/NC_000913.3). The RBS was located to facilitate effective knockdown of *wecD* expression at translation by asRNA hybridization. Based on previous knowledge, the RBS is located upstream from the gene and may contain the entire or part of the Shine-Dalgarno (SD) sequence, which is located within 6-7 base pair (bp) of the start codon of the gene (http://parts.igem.org/Help:Ribosome_Binding_Sites/Mechanism). Ribosomal protein S1 binds to sequences enriched with adenine bases found further upstream of the SD sequence (http://parts.igem.org/Ribosome_Binding_Sites/Design). Based on these characteristics, a section of 60 bps upstream of *wecD* was selected to use as the antisense insert sequence for the plasmid. The sequence selected is exclusive to the region where the tail of the previous gene *rffH* and beginning of *wecD* overlapped. Since asRNA silencing of expression at translation is more effective when targeting the RBS of a gene, it is likely that only *wecD* is silenced. To ensure the complementarity of the antisense transcript with *wecD* mRNA, a reverse complement sequence was generated from the coding strand. The 5′ and 3′ ends of the insert sequences were altered to complement restriction enzyme sites in the plasmid to generate sticky ends and facilitate ligation. The insert DNA sequences and features used in construction of the *wecD* mRNA antisense plasmid are summarized in Table 2.

TABLE 2 Insert DNA sequences and features used in construction of the *wecD* mRNA antisense plasmid.

Inserts	Restriction enzyme	Insert Sequence* (5′ to 3′)	Product size (Base pair)
*wecD*as60f (forward *wecD*)	NcoI	catggTGGCGCGGACGGGCACGAAGTAACTCCAGCAGATATTGGCCGTAGCCAGTTTTCc	60
*wecD*as60b (reverse *wecD*)	XhoI	tcgagGAAAACTGGCTACGGCCAATATCTGCTGGAGTTACTTCGTGCCCGTCCGCGCCAc	

Plasmid isolation from MG1655. Plasmids pZ and pHN678 were provided within MG1655 from the Ramey culture collection. Isolated MG1655 colonies containing either one of these plasmids were grown in 5 mL of overnight culture, with 30 µg/mL chloramphenicol, on a shaker at 37 °C. The entire culture was then used for plasmid isolation using the PureLink Quick Plasmid DNA Miniprep Kits (Invitrogen, K2100) according to the manufacturer's centrifugation protocol. Plasmid samples were stored at -20 °C until use.

Oligonucleotide generation, phosphorylation, and ligation into pHN678. The annealing of *wecD*as60f and *wecD*as60b were done according to the Protocol for Annealing Oligonucleotides (Sigma-Aldrich). The annealed samples were stored at -20 °C until use. Oligonucleotide phosphorylation on the annealed sample was done according to the Protocol for Non-radioactive Phosphorylation with T4 PNK (NEB, M0201S). Ligation of the phosphorylated *wecD* insert strands into the vector plasmid, pHN678, was done according to the protocol adapted from the Ligation Protocol with T4 DNA Ligase (NEB, M0202). The ligation reaction mixture was incubated at room temperature for 90 minutes before being used in transformation. Aliquots of this ligation product, pKAT*wecD*, was stored at -20 °C.

Transformation into competent cells. The Hancock Lab's protocol for $CaCl_2$ transformation of *E. coli* was used to create competent MG1655 cells which were then stored at -80 °C until use (14). The frozen aliquots were thawed and subsequent heat-shocking steps from the same protocol were used to transform pZ, pHN678, or pKAT*wecD* into competent MG1655 cells (14). The cells were then plated onto LB agar plates containing 30 µg/mL chloramphenicol for selection of pZ, pHN678, or pKAT*wecD* containing transformants, and incubated at 37 °C overnight. From plates containing MG1655 colonies transformed with pKAT*wecD*, isolated colonies were randomly selected and inoculated into 5 mL of LB broth with 30 µg/mL chloramphenicol to make overnight cultures. Plasmids were isolated from the culture using the PureLink Quick Plasmid DNA Miniprep Kits (Invitrogen, K2100) according to the manufacturer's centrifugation protocol.

Sequencing for confirmation of proper pKAT*wecD* design. Isolated pKAT*wecD* plasmid (50 ng/µL) from transformants, with asRNA2 primer (10 pmol/µL) from *Chen et al.*(2015), were sent out for sanger sequencing at UBC's NAPS Unit (15). Betaine additive and dGTP chemistry was requested to help resolve common sequencing issues such as G-C rich templates and hairpin structures, especially with the presence of a paired termini from the plasmid. Confirmation of the sequencing results were done by using the NCBI Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Growth rate of uninduced and IPTG-induced *E. coli* BW25113, JW5597-1, MG1655, and MG1655 harboring pZ, pHN678, or pKAT*wecD*. To assess cell growth rate with inhibition of *wecD*, 50 mL LB overnight cultures of BW25113, JW5597-1 with 50 µg/mL kanamycin, WT MG1655, and MG1655 (with pZ, pHN678, or pKAT*wecD*) with 30 µg/mL chloramphenicol were made. The overnight cultures were inoculated into fresh 25 mL LB (1:125 dilution or OD_{600} less than 0.05), with the appropriate antibiotics, shaken at 37 °C, and grown up to an OD_{600} of approximately 0.2. An aliquot of each culture was transferred to sterile test tubes and

induced with 1 mM IPTG. Using LB as the blank, 200 µL of each sample (uninduced and 1 mM IPTG induced) was added into a 96-well plate in triplicates and the OD_{600} readings were monitored with the Ultraspec 3000 UV/visible Spectrophotometer. Readings were taken every 20 minutes until late log phase was reached.

Microscopic observation of uninduced and IPTG-induced MG1655 harboring pZ. To visualize cell growth, wet mounts were prepared and observed at 250 minutes post-induction using the Unilux-12 Microscope (Kyowa) at 1000x magnification with oil immersion.

Bacteriophage T7 Infectivity assay on the growth rate of *E. coli* BW25113, JW5597-1, MG1655, and MG1655 harboring pZ, pHN678, or pKAT*wecD*. Bacteriophage T7 infectivity on the cell growth rate, with inhibition of *wecD*, was assessed at 0.25 and 0.50 multiplicity of infection (MOI). 50 mL LB overnight cultures of BW25113, JW5597-1 with 50 µg/mL kanamycin, WT MG1655, and MG1655 (with pZ, pHN678, or pKAT*wecD*) with 30 µg/mL chloramphenicol were made. The overnight cultures were inoculated into fresh 25 mL LB (1:125 dilution or OD_{600} less than 0.05), with the appropriate antibiotics, shaken at 37 °C, and grown up to an OD_{600} of approximately 0.2. An aliquot of each culture was transferred to sterile test tubes and bacteriophage T7 was added to achieve 0.25 and 0.50 MOI. Induction with 1 mM IPTG was then done right after. Using LB as the blank, 200 µL of each sample was added into a 96-well plate in triplicates and the OD_{600} readings were monitored with the Ultrospec 3000 UV/Visible Spectrophotometer. For the first assay, readings were recorded every 15 minutes and after 100 minutes, readings were recorded every 30 minutes (summarized in the supplementary section). For the second assay, readings were recorded every 20 minutes until the same trend as the first assay was observed.

RESULTS

Sequencing of pKAT*wecD* confirms proper antisense plasmid design. The construction of pKAT*wecD* allowed gene silencing of *wecD* via antisense RNA. The forward and reverse DNA strands (summarized in Table 2), designed for the *wecD* antisense component of pHN678, were annealed together to generate a double-stranded oligonucleotide. This oligonucleotide was then phosphorylated before ligation into pHN678 to generate pKAT*wecD*. This plasmid was transformed into MG1655 and then isolated for sequencing. Sanger sequencing results of pKAT*wecD* and the *wecD*as60f insert sequence were aligned with each other using NCBI BLAST. The BLAST results (Fig. S4) showed exact alignment between the two sequences which confirms that the *wecD* antisense sequence was properly inserted into pHN678. A diagram of pKAT*wecD* and the resulting paired termini *wecD* mRNA antisense insert sequence is shown in Figure 1.

Due to limitations imposed on the experiment by facility restrictions and time needed to raise custom antibodies, a direct method of measuring WecD protein expression using techniques such as Western blot was beyond the scope of this experiment.

IPTG induction of pZ yields an elongated morphology in MG1655. pZ, a derivative of pHN678 containing a *ftsZ* antisense insert, was used as a function control plasmid for asRNA expression (16). Upon IPTG induction pZ inhibits production of FtsZ, a highly conserved protein that is key for bacterial cellular division. Therefore, *ftsZ* mutants are unable to divide, exhibiting an elongated morphology and increased cell death (16). In our plasmid function control experiment, both the uninduced and IPTG-induced WT MG1655 had rod-shaped morphology which indicates that bacterial morphology was not affected by IPTG. Uninduced MG1655/pZ cells exhibited normal rod-shaped morphology whereas IPTG-induced cells exhibited an elongated morphology (Fig. 2). Based on this observation, we can confirm that the asRNA sequence regulated by the strong synthetic promoter *trc* was expressed upon IPTG induction. Once expressed, the asRNA sequence, stabilized by the paired-termini secondary structure was able to silence *ftsZ* expression. Given that pKAT*wecD* is identical to pZ with the exception of the asRNA sequence, we can expect that the asRNA sequence will also be expressed upon IPTG induction and silence *wecD* expression. This test also served to confirm the potency of the amount of IPTG used for induction.

IPTG slightly hindered the growth of WT MG1655, pZ, pHN678, and pKAT*wecD*. Expression of the asRNA sequence in pKAT*wecD* is induced by IPTG; however the effect of IPTG on the growth rates of the strains used is unclear. In the T7 infectivity assay, strains are inoculated with T7 bacteriophage and the change in optical density at 600nm (OD_{600}) over time is used to measure susceptibility to T7 infection. It was expected that the OD_{600} would increase as the cells grew, then decrease as T7 infection caused the cells to lyse. Therefore, potential changes in growth rate caused by IPTG will alter the OD_{600} of the

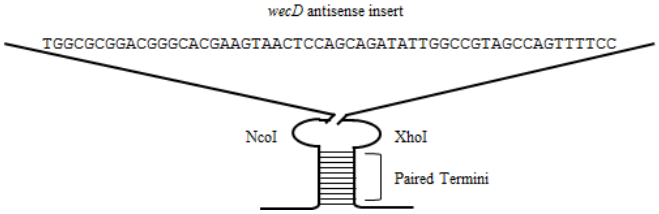

FIG. 1 Transcribed *wecD* antisense RNA stabilized by paired termini.

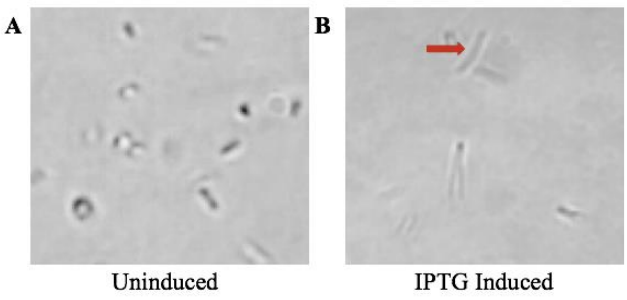

FIG. 2 IPTG induction altered cell morphology in *E. coli* MG1655 harboring pZ. (A) Uninduced. **(B)** Induced with 1 mM IPTG.

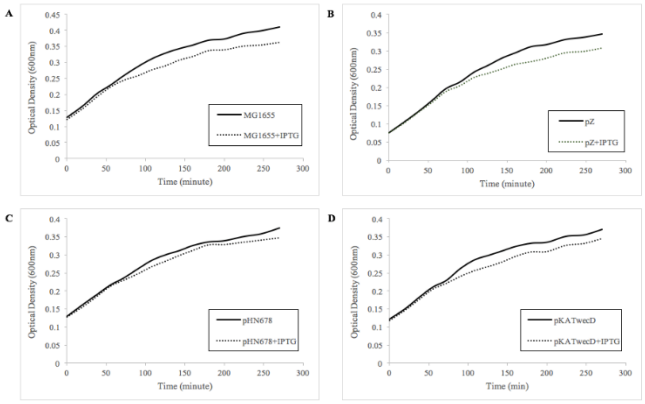

FIG. 3 The effect of 1 mM IPTG on the growth rate of WT MG1655 and pZ, pHN678 and pKAT*wecD* transformants. (A) WT MG1655 strain. **(B)** MG1655 transformed with pZ plasmid. **(C)** MG1655 transformed with pHN678 plasmid. **(D)** MG1655 transformed with pKAT*wecD* plasmid. The optical density of cultures was measured at 600 nm for 270 minutes.

sample. Optical density at 600 nm was used to monitor growth rates of *E. coli* WT MG1655, pZ transformant, pHN678 transformant, and pKAT*wecD* transformant, to determine the effect of IPTG on growth rate. Figure 3 compares the growth trend of uninduced and 1 mM IPTG-induced cells of the four strains. In all four strains, IPTG induction seems to slightly hinder the growth rate however we expected to see a more notable and slower growth rate of IPTG-induced pZ strain due to antisense silencing of *ftsZ* leading to inhibition of cellular division (Fig. 3). Instead, a slower growth rate was somewhat more notable for both WT MG1655 and pZ. As the growth curve was only done once, we can only conclude that all four strains were slightly hindered upon IPTG induction but more replicates would be needed in order to conduct statistical analysis.

Gene silencing from *wecD* genomic deletion sensitized *E. coli* cells to bacteriophage T7 infection. The bacteriophage T7 infectivity assay was designed to compare susceptibilities of *E. coli* wildtype and *wecD* silenced mutant strains to T7 phage. Optical density at 600 nm was used to monitor cell death due to bacteriophage T7 infection in *E. coli* cultures and to determine the effect of *wecD* genomic deletion in bacteriophage T7 infectivity.

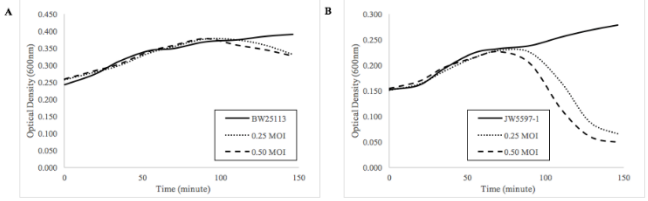

FIG. 4 The effect of *wecD* genomic deletion on bacteriophage T7 infectivity at 0.25 and 0.5 MOI. (A) Parent/WT BW25113 strain. **(B)** *wecD* genomic deletion JW5597-1 strain. The optical density of cultures was measured at 600 nm for 146 minutes.

Figure 4 shows the bacteriophage T7 infectivity assay results in WT BW25113 and *wecD* genomic deletion JW5597-1 at 0.25 and 0.5 MOI. As expected, a steeper decline in cell density was observed at 0.5 MOI compared to 0.25 MOI in both WT and mutant strains, indicating faster cell death rates at higher bacteriophage T7 concentration. At around 70 minutes, cell growth of BW25113 started slowing down gradually, while cell density of JW5597-1 dropped rapidly. The same trend was also observed when the assay was replicated (Fig. S2). This indicates that bacteriophage T7 has a greater impact on cell growth in the *wecD* genomic deletion strain as compared to the WT BW25113 strain. At the end of the measurement at 146 minutes, the cell survival percentage in 0.5 MOI infected culture relative to the uninfected culture was 83.6% in BW25113 (from OD_{600} 0.327 and 0.392) and 17.3% in JW5597-1 (from OD_{600} 0.048 and 0.279). A 4.8-fold decrease in cell survival rate, and therefore a 4.8-fold increase in bacteriophage T7 infectivity, was observed in the *wecD* genomic deletion JW5597-1 strain relative to the WT BW25113 strain. Therefore, *wecD* genomic deletion dramatically sensitized *E. coli* to bacteriophage T7 infection.

Gene silencing from *wecD* asRNA knockdown also sensitized *E. coli* cells to bacteriophage T7 infection. In the bacteriophage T7 infectivity assay, optical density at 600 nm was used to monitor cell death due to bacteriophage T7 infection in *E. coli* cell cultures, and to determine the effect of *wecD* asRNA knockdown on phage infectivity. Figure 5 shows the bacteriophage T7 infectivity assay results, at 0.25 and 0.5 MOI, in the WT MG1655 and transformant strains harboring pHN678 and pKAT*wecD*. Overall, 0.5 MOI resulted in a greater decline in cell density in all strains and conditions compared to 0.25 MOI. At approximately 90 to 100 minutes, cell growth started

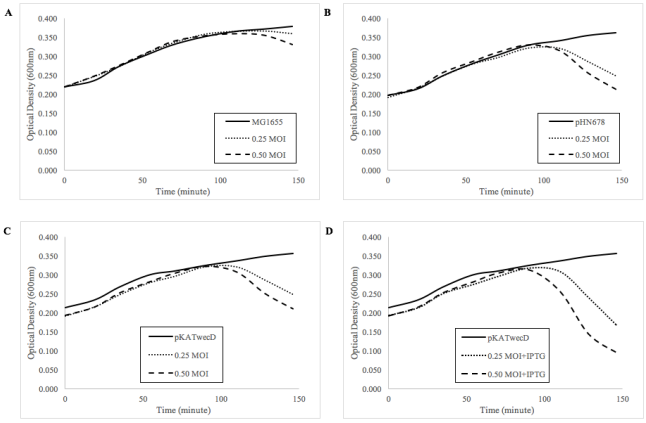

FIG. 5 The effect of *wecD* asRNA knockdown on bacteriophage T7 infectivity at 0.25 and 0.5 MOI. (A) WT MG1655 strain. **(B)** MG1655 transformed with pHN678 plasmid. **(C)** MG1655 transformed with pKAT*wecD* plasmid, without IPTG induction. **(D)** MG1655 transformed with pKAT*wecD* plasmid, with 1mM IPTG induction. The optical density of cultures was measured at 600 nm for 145 minutes.

slowing down and decreasing gradually. By 146 minutes, the cell survival rate in MG1655, under 0.5 MOI relative to the uninfected group, was observed to be 87.3% (from OD_{600} 0.331 in 0.379), as shown in Figure 5. Similarly, the survival rate of pHN678 was calculated to be 59.0% (from OD_{600} 0.214 in 0.363) at the end of the measurement (Fig. 5). Figure 5 compares bacteriophage T7 infectivity in pKAT*wecD* transformant without and with 1 mM of IPTG induction. Under induction, a much lower survival rate (26.6%) was seen compared to the uninduced group (59.0%). In addition, the IPTG-induced pKAT*wecD* transformant exhibited a 3.3-fold decrease in survival rate, indicating that asRNA knockdown of *wecD* also significantly sensitized *E. coli* to bacteriophage T7 infection. Additionally, the same trend was observed when the assay was replicated (Fig. S3).

DISCUSSION

In this experiment, we investigated the role of WecD on phage resistance in *E. coli* by silencing *wecD* with asRNA knockdown. Although WecD expression was not measured directly using techniques such as Western blot in our study, the expression of the asRNA sequence contained in pKAT*wecD* was indirectly verified using a phenotype comparison with pZ instead. Since the original plasmid for both pZ and pKAT*wecD* was pHN678, if pZ was able to silence the expression of *ftsZ* and result in visible elongation of *E. coli,* we expected that the asRNA sequence to *wecD* would also be expressed. Additionally, the effect of *wecD* knockdown was confirmed by comparison to strain JW5597-1, which has a chromosomal deletion for *wecD*.

As mentioned previously, McMahon *et al.* observed in *S. marcescens* that silencing *wecD* resulted in a hypervesiculation phenotype of the cells (6). We were unable to confirm the results of McMahon *et al.* given the lack of access to *S. marcescens*. However, since ECA is conserved among all members of the Enterobacteriaceae family, we expected that the phenotype of silencing *wecD* in *S. marcescens* would also be observed in *E. coli* (4). In addition, Kulp and Kuehn observed that OMV production increases during the onset of phage adhesions to the cell surface and proposed that this mechanism helps bacteria avoid phage infection (7). Based on this, we hypothesized that silencing *wecD* would increase resistance to bacteriophage T7 infection in *E. coli.* The proposed mechanism of this was that silencing *wecD* expression will disrupt ECA biosynthesis and increase OMV production. Due to time restrictions and equipment limitations, we were unable to directly measure OMV production; instead we focused on the expected observation as a result of the proposed mechanism. If silencing of *wecD* expression in *E. coli* increased OMV production, then the cell's ability to resist phage infective would be expected to increase.

A phage infectivity assay was used to determine the susceptibility of *E. coli* to bacteriophage T7 infection. Contrary to our hypothesis, both the *wecD* genomic deletion and the *wecD* asRNA knockdown increased *E. coli* sensitivity to bacteriophage T7 infection. From comparison of the WT strains in both experimental model systems, BW25113 and MG1655 showed very similar survival rates of 83.6% and 87.3%, respectively. The fold increase of cell sensitivity to bacteriophage T7 relative to their respective WT strains was 4.8 in the *wecD* genomic deletion JW5597-1 strain and 3.3 in the *wecD* asRNA knockdown strain. This observed sensitivity in both experimental model systems, contrary to our original hypothesis, could be explained by many other hypotheses that are plausible. We suspect that since the only difference in culture conditions between controls, MG1655, BW25113, and the test strains was that the test strains were cultured in the presence of antibiotics, it is possible the presence of antibiotics may also increase sensitivity to bacteriophage T7 infections in *E. coli*. To observe the effect of *wecD* knockdown independently of antibiotics, we used a plasmid with IPTG induction. The uninduced plasmid was used as a control for pKAT*wecD* and cultured in the presence of chloramphenicol. BW25113, the control for JW5575-1 knockout strain, was not cultured in the presence of kanamycin. Since IPTG-induced pKAT*wecD* led to a further increase in sensitivity compared to the uninduced control, we concluded that silencing *wecD* expression also played a role in increasing the susceptibility of *E. coli* to T7 bacteriophage infection. We suspected that the absence of ECA may also induce membrane stress in the OM and compromise the physical protection of the cell.

To explain the observed increase in phage sensitivity upon *wecD* suppression, we suspect that membrane stress induced by the disruption of ECA biosynthesis, as a result of silencing *wecD* expression, may compromise the physical protection of the cell from bacteriophage infections. As mentioned previously, the function of ECA is poorly characterized but it has been linked to cell resistance to environmental conditions (17). As a surface antigen in the outer leaflet of the OM in Gram-negative enteric bacteria, ECA may play a vital role in the cellular protection from environmental factors including bacteriophage infection (18). Furthermore, Castelli *et al*. determined that disruption of ECA biosynthesis induced membrane stress (19). Therefore, the absence of ECA in *wecD* mutants may compromise the ability of

the membrane to protect the cell from the environment. The major limitation of our experiment was that the bacteriophage T7 infectivity assay only tested our initial hypothesis that silencing *wecD* will increase *E. coli* resistance to T7 infection. Due to time constraints and the lack of equipment to culture large volumes of cells, we were unable to test proposed mechanism of increased OMV production. Therefore, we cannot verify whether silencing *wecD* in fact increases OMV production in *E. coli*. If the hypothesized response of hypervesiculation due to silencing *wecD* expression is absent, then the induction of membrane stress from the absence of ECA may instead contribute to sensitizing *E. coli* to bacteriophage T7 infection by compromising the physical protection of the cell.

Alternatively, we suspect that the observed increase in susceptibility of WecD-deficient cells to bacteriophage infection as compared to WT cells could be due to the presence of antibiotics, which may increase sensitivity to bacteriophage T7 infections in *E. coli*. From our phage infectivity assay, we observed that the presence of pHN678 or uninduced pKAT*wecD* plasmid in MG1655 appears to increase *E. coli* susceptibility to bacteriophage T7 infection compared to WT MG1655 (Fig. 5). pHN678 only contained p15A ori, *lac o,* complementary sequences for paired termini formation, terminator sequence, *lacI*, the Chloramphenicol resistance gene *cmr*, and the respective promoters. As a result, pHN678 is unlikely to affect phage infectivity. The culture conditions between WT MG1655 and plasmid possessing MG1655 were identical with the exception that both plasmid possessing MG1655 strains were cultured in the presence of chloramphenicol as the selection mechanism for positively transformed cells. Chloramphenicol acetyltransferase (CAT) is the enzyme responsible for chloramphenicol resistance in bacteria by preventing the antibiotic from binding onto ribosomes (20). We were unable to determine the extent of chloramphenicol resistance in our plasmid host strains therefore it is plausible that the presence of chloramphenicol induced cellular stress which may increased susceptibility to bacteriophage T7 infection. As mentioned previously, *wecD* genomic deletion JW5597-1 also increased *E. coli* sensitivity to bacteriophage T7 infection and it was cultured in kanamycin as the selection mechanism. Therefore, it is also unclear whether the increased sensitivity to bacteriophage T7 was solely a result of *wecD* genomic deletion or the combination of a lowered host resistance due to kanamycin and *wecD* genomic deletion. Nonetheless, IPTG induction of pKAT*wecD* further reduced the cell density (Fig. 5), which supports the observation that silencing *wecD* increased *E. coli* sensitivity to bacteriophage T7 infection. Additionally, and per our expectation that a genomic deletion is a more effective method than asRNA knockdown for mitigating gene expression, *wecD* genomic deletion was observed to increase *E. coli* sensitivity to bacteriophage T7 infection more than *wecD* asRNA knockdown.

As previously mentioned in our growth curve analysis, we did not observe a significant difference in the growth rate of WT MG1655 and MG1655 transformed with pHN678 or pKAT*wecD* (Fig. 3). Thus, we did not add IPTG as a part of the negative control for the phage infectivity assay. The variations in growth rates observed could be attributed to experimental method errors of utilizing 96-well plates which are subjected to edge effects and volume inaccuracies. Due to time constraints, the growth rate analysis was only conducted once and more replicates will contribute statistical significance to the mean growth rate observed. We also recognize that there is a possibility that IPTG affected the susceptibility of *E. coli* to bacteriophage T7 infection or disrupted the proposed mechanism of increased OMV production. The *wecD* genomic deletion strain JW5597-1 was cultured in the absence of IPTG and produced comparable results to IPTG-induced pKAT*wecD*. Therefore, IPTG likely did not cause disruptions to cellular functions. It is more likely that the increased sensitivity to bacteriophage T7 infection is the result of silencing *wecD* expression.

In conclusion, silencing of gene expression via *wecD* genomic deletion and *wecD* asRNA knockdown dramatically sensitized *E. coli* cells to bacteriophage T7 infection. Although we could not directly verify whether hypervesiculation was present as a result of silencing *wecD* expression, we suspected that the envelope stress and disruption to the OM caused by the absence of ECA may have increased the sensitivity to T7 bacteriophage infection.

FUTURE DIRECTIONS

As mentioned previously, we hypothesized that silencing *wecD* and thereby disrupting ECA biosynthesis would increase OMV production and therefore increase bacteriophage T7 resistance of *E.coli*. However, contrary to our hypothesis, both the *wecD* genomic deletion and the *wecD* asRNA knockdown strains instead showed increased sensitivity to bacteriophage T7 infection. To further test our findings, it would be important to determine whether or not silencing *wecD* expression leads to a hypervesiculation phenotype in the T7 infected cells. We were unable to directly measure OMV production due to time and technical constraints. If sufficient amounts of cells

can be cultured, ultracentrifugation may be used by future groups to isolate OMV particles. Additionally, KDO assay can be performed on the crude OMV isolate to better separate the LPS containing OMV from other cellular debris.

In order to select for certain antibiotic-resistance or transformed cells, the transformed cells used in the present report were cultured in the presence of antibiotic selection. However, *E. coli* MG1655 transformed with either pHN678 or pKAT*wecD* appeared to have increased sensitivity to bacteriophage T7 infection without IPTG induction. This indicated that it will be important to verify the impact of antibiotic on sensitivity to bacteriophage T7 infection in future work. Future groups may vary the concentration of the antibiotics in transformed cultures to determine the impact it may have on sensitivity to bacteriophage T7 infection. Alternatively, WT MG1655 and BW25113 could be cultured in the presence of chloramphenicol and kanamycin, respectively, at sub-MIC (minimum inhibitory concentration) level and infected with bacteriophage T7 to isolate the effect of antibiotics on sensitivity to bacteriophage T7 infection. Similarly, the effect of IPTG could be isolated using the same method.

To assess the sensitivity of WecD-deficient *E. coli* cells to bacteriophage T7 infection, we infected our *E. coli* culture at different MOI. However, we did not vary the concentration of IPTG used to induce pKAT*wecD* to express the asRNA transcript and silence *wecD* expression. It was observed that when induced, the cells became more sensitive to bacteriophage T7 infection. To reinforce this observation, IPTG concentration could be varied to determine whether sensitivity to bacteriophage T7 infection changed with the level of induction.

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REFERENCES

1. **Dy, RL, Richter, C, Salmond, GPC, Fineran, PC.** 2014. Remarkable mechanisms in microbes to resist phage infections. Annual Review of Virology. **1:**307-331.
2. **Housby, JN, Mann, NH.** Phage therapy. Drug Discov Today. **14:**536-540.
3. **Örmälä, A, Jalasvuori, M.** 2013. Phage therapy: Should bacterial resistance to phages be a concern, even in the long run? Bacteriophage. **3:**e24219.
4. **Kuhn, H.** 1988. ECA, the enterobacterial common antigen. FEMS Microbiol Lett. **54:**195-222.
5. **Hung, M, Rangarajan, E, Munger, C, Nadeau, G, Sulea, T, Matte, A.** 2006. Crystal structure of TDP-fucosamine acetyltransferase (WecD) from *Escherichia coli*, an enzyme required for enterobacterial common antigen synthesis. J Bacteriol. **188:**5606-5617.
6. **McMahon, KJ, Castelli, ME, Vescovi, EG, Feldman, MF.** 2012. Biogenesis of outer membrane vesicles in *Serratia marcescens* is thermoregulated and can be induced by activation of the Rcs phosphorelay system. J Bacteriol. **194:**3241.
7. **Kulp, A, Kuehn, MJ.** 2010. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. Annu Rev Microbiol. **64:**163-184.
8. **Loeb, MR, Kilner, J.** 1978. Release of a special fraction of the outer membrane from both growing and phage T4-infected *Escherichia coli*. B Biochim Biophys Acta. **514:**117.
9. **Hardman, B, Hunt, D, Mojaab, D, Naor, A.** 2016. Sub-lethal gentamicin treatment of *Escherichia coli* UB1005 induces the release of soluble factors that reduce susceptibility to T7 bacteriophage infection. JEMI. **21:**128-133.
10. **Bleackley, J, Cooper, J, Kaminski, M, Sandilands, S.** 2009. The reduction of T7 phage adsorption in *Escherichia coli* B23 cells treated with sub-lethal levels of kanamycin. JEMI. **13:**89-92.
11. **Gu, E, Nguyen, D, Shah, N.** 2011. Capsular polysaccharide has a minor role on streptomycin-induced reduction of T7 phage adsorption to *Escherichia coli*. JEMI. **15:**47-51.
12. **Waters, LS, Storz, G.** 2009. Regulatory RNAs in bacteria. Cell. **136:**615-628.
13. **Nakashima, N, Tamura, T, Good, L.** 2006. Paired termini stabilize antisense RNAs and enhance conditional gene silencing in *Escherichia coli*. Nucleic Acids Res. **34:**e138.
14. **Hancock, REW.** 1999. Hancock Laboratory Methods.
15. **Chen, D, Lam, A, Lin, S, Wong, J.** 2015. Antisense RNA targeting the first periplasmic domain of YidC appears to reduce cell growth at 30 degrees celsius but does not induce a filamentous phenotype. JEMI. **19**.
16. **Boberek, JM, Stach, J, Good, L.** 2010. Genetic evidence for inhibition of bacterial division protein FtsZ by berberine. PloS One. **5:**e13745.
17. **Goździewicz, TK, Łukasiewicz, J, Ługowski, C.** 2015. The structure and significance of enterobacterial common antigen (ECA). Postepy Higieny i Medycyny Dos□wiadczalnej (Online). **69:**1003.
18. **Zhang, G, Meredith, TC, Kahne, D.** 2013. On the essentiality of lipopolysaccharide to Gram-negative bacteria. Curr Opin Microbiol. **16:**779.
19. **Castelli, ME, Véscovi, EG.** 2011. The Rcs signal transduction pathway is triggered by enterobacterial common antigen structure alterations in *Serratia marcescens*. J Bacteriol. **193:**63-74.
20. **Hartley, BS, Packman, LC, Burleigh, BD, Dell, A, Shaw, WV, Morris, HR.** 1979. Primary structure of a chloramphenicol acetyltransferase specified by R plasmids. Nature. **282:**870-872.