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Steps towards construction of a plasmid to express antisense RNA directed at the ribosomal binding site of *wbbL* in *Escherichia coli* DFB1655 L9

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SUMMARY The O antigen oligosaccharide in the outer membrane of *Escherichia coli* has been found to confer resistance against T4 bacteriophage, thereby preventing cell lysis. O antigen production is regulated by genes found in the *rfb* gene cluster, including w*bbL.* which encodes for WbbL, a rhamnose transferase. Chow *et al*. developed an antisense RNA (asRNA) silencing model targeting *wbbL* to determine if silencing this gene in *E. coli* K-12 substrain, DFB1655 L9, would increase susceptibility to T4 bacteriophage but did not test functionality. Our project builds upon the work of Chow *et al*. by adapting and improving the screening process of their asRNA construct. Our approach is to insert the adapted asRNA into an empty pHN678 plasmid vector, transform *Escherichia coli* TOP10 competent cells with our plasmid, and screen for the presence of our insert through gel electrophoresis. Through screening our colonies for the ligated plasmid on a gel, we found evidence for the presence of our insert. However, attempts at Sanger sequencing of pMOD-wbbL(a) to confirm presence of the insert were inconclusive. Thus, further experiments are needed to test our asRNA silencing model targeting *wbbL,* with the aim of determining if this affects O antigen production or T4 bacteriophage resistance.

INTRODUCTION

Bacteria have evolved mechanisms of resistance against bacteriophages, viruses that specifically infect bacteria (1). T4 bacteriophage is a member of the Myoviridae - a complex family of tailed phages- that is capable of infecting and inducing lysis in *E. coli* (2). T4 phage infection is initiated by the interaction between the tail fibers of the bacteriophage and specific receptors on the surface of *E. coli* (3). To prevent infection by T4, Gram negative *Escherichia coli* have developed mechanisms of resistance. For example, within the lipopolysaccharide (LPS) in the outer membrane of *E. coli* is a repetitive oligosaccharide unit called the O antigen that can mediate resistance (1). The O antigen is a restriction factor and a point of interaction with certain bacteriophages, including the T4 bacteriophage (4). Although the exact mechanism by which O antigen blocks T4 infection of *E. coli* is unknown, several models have been suggested. One example is the steric hindrance model, where O antigen may physically block T4 tail fibers interacting with and binding to cell surface receptors (1). Other methods investigate the role of O antigen as a soluble factor that interacts with T4 bacteriophage, as well as its role in T4 replication within *E. coli* (1).

The production of O antigen is regulated by genes from the *rfb* gene cluster, including *wbbL* which encodes for WbbL, a rhamnose transferase (4). *E. coli* K-12 is a commonly used lab strain that has lost the ability to produce O antigen due to mutations that have accumulated over time within *rfb* (1). MG1655 is a substrain of *E. coli* K-12 with an IS5 insertion element disrupting *wbbL* expression, preventing O antigen production and increasing T4 bacteriophage susceptibility (5, 6). To study O antigen production and its effects on T4 bacteriophage resistance in *E. coli*, another substrain can be produced by inserting an intact *rfb* gene locus into MG1655, thereby restoring O antigen production and potentially T4 bacteriophage resistance (2). This resulting substrain is referred to as DFB1655 L9 (6).

Silencing *wbbL* allows us to differentiate whether the increase in resistance to T4 in DFB1655 L9 is due to the restored production of WbbL, or potential polar effects arising due to the homologous recombination of *wbbL* in DFB1655 L9 (7). This gene can be silenced

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using antisense RNA (asRNA), a type of non-coding RNA that functions primarily in regulating the expression of genes (8). DNA is transcribed to generate a short strand of asRNA approximately 15-50 nucleotides long with bases that are complementary to the mRNA transcript of a certain gene (9). The asRNA hybridizes with the mRNA to create a double stranded RNA (dsRNA) molecule that is translationally inactive (8). Although asRNAs occur naturally in cells to regulate the expression of genes from various pathways at a post transcriptional level, they can also be created in the lab. DNA coding for the asRNA can be ligated into an antisense expression vector such as pHN678 and then transformed into competent cells where the insert will be expressed, leading to the production of asRNA (10).

In their 2019 study, Chow *et al*. developed an RNA antisense silencing model targeting *wbbL* in order to determine whether silencing this gene in *E. coli* DFB1655 L9 would increase susceptibility to T4 bacteriophage (11). They found that transforming DFB1655 L9 with asRNA targeting *wbbL* did not affect T4 bacteriophage resistance (11). When exposed to T4 bacteriophage at a multiplicity of infection (MOI) of 3, DFB1655 L9 with asRNA was found to be resistant to approximately the same level as DFB1655 L9 without asRNA targeting *wbbL*, and MG1655 was confirmed to be susceptible (11). However, Chow *et al.* did not measure WbbL production in the transformed DFB1655 L9 to verify that their construct silenced *wbbL*. In addition, recreation of the construct was necessary because pCODA-wbbL(a) could not be found in freezer stocks. Our study builds upon this by recreating the RNA antisense silencing model developed by Chow *et al.* and potentially improving the screening process via the destruction of the restriction enzyme cleavage sites following insertion of the antisense sequence.

METHODS AND MATERIALS

Bacterial strains used in this study. *E. coli* K-12 substrains MG1655 and DFB1655 L9, available in the MICB 401 laboratory, were originally a gift from Dr. Douglas F. Browning of the Henderson laboratory situated in the University of Birmingham (6). The MG1655 strain has an IS5 insertion element within the *wbbL* gene sequence, rendering it incapable of O antigen production (6). The DFB1655 L9 strain was generated by insertion of an intact *wbbL* gene upstream of the IS5 insertion element in MG1655, regenerating the complete *rfb* gene cluster (6). Hence, DFB1655 L9 is capable of O antigen production (6). Competent *E. coli* One Shot™ TOP10 cells were obtained from ThermoFisher Scientific (cat no. 18265017 and C404010 respectively). Both MG1655 and DFB1655 L9 cells were streaked onto 1.5% Luria-Bertani (LB) agar plates, grown overnight at 37°C to obtain isolated colonies, and subsequently placed at 4°C for long-term storage.

Preparing competent cell stocks of MG1655 and DFB1655 L9. This protocol was adapted from the American Society of Microbiology (ASM) (12). One isolated colony from each of the MG1655 and DFB1655 L9 plates was inoculated into a 5 mL LB broth aliquot and placed in a 37°C shaker at 225 RPM overnight. 1 mL of each overnight culture was then subcultured into an Erlenmeyer flask containing 100 mL of LB broth, subsequently placed in the 37°C shaker. OD_{600} readings of subcultures were obtained every 30 minutes until the cultures reached log phase, and the flask began to look turbid. Subcultures were then separated into Oak Ridge tubes and placed on ice for 10 minutes. They were then centrifuged at 4,000 g and 4°C for 5 minutes, followed by resuspension in ice-cold sterile 60mM $CaCl_2$. The cultures were placed back on ice for 30 minutes and centrifuged again with the same setting. They were then resuspended in 5 mL of 60mM $CaCl_2$ + 15% glycerol solution and stored at 4°C for up to 24 hours to increase transformation efficiency. Lastly, the cells were dispensed into microfuge tubes in 250 µL aliquots and placed at -70°C for long-term storage.

Transformation of competent MG1655 and DFB1655 L9 with pHN678. The protocol for transformation was adapted from ASM (12). Aliquots of the pHN678 plasmid were pipetted into microfuge tubes containing MG1655 and DFB1655 L9. After mixing gently, the tubes were incubated on ice for 30 minutes. The tubes were then transferred to a 42°C water bath for 2 minutes before being placed back on ice for 5 minutes. The cells were then transferred from the microfuge tubes into test tubes containing 1 mL of Super Optimal broth with

Catabolite repression (S.O.C) medium, and then incubated at 37°C for 1 hour in a 225 rpm shaker. Cells were then plated on LB medium supplemented with 25 µg/mL chloramphenicol, and incubated overnight at 37°C.

Designing *wbbL* asRNA. The asRNA design was adapted from Chow *et al.* (11). The *wbbL* gene sequence was obtained from the NCBI database Gene ID 4056030, and the molecular biology software SnapGene™ was used to view the gene sequence and verify that the Chow *et al.* asRNA was designed as intended (13). The backbone of the antisense primer designs were left unchanged from the Chow *et al.* design following this verification step. Next, in order to introduce a restriction site that would destroy the NcoI and XhoI cut sites following insertion, the guanine nucleotide in the 5th position was switched to a cytosine in both the forward and the reverse primers. The complementary cytosine nucleotide at the end of each primer sequence was also switched to a guanine to reflect the change made in the 5th position. A comparison of the modified design and the original Chow *et al.* design is shown in Table1.

TABLE. 1 Primer sequences for antisense construction. Annealed primers designed by Chow *et al*. and our modified asRNA model. Bolded red nucleotides indicate differences.

Antisense Design	Primer Sequences
Chow *et al.*	5′-catg**g**TATACCATTTCAATGTTCTTCAGTAATAAAATTAACTAGTTCATCAAA**c**-3′ 3′-**c**ATATGGTAAAGTTACAAGAAGTCATTATTTTAATTGATCAAGTAGTTT**g**agct-5′
Modified	5′-catg**c**TATACCATTTCAATGTTCTTCAGTAATAAAATTAACTAGTTCATCAAA**g**-3′ 3′-**g**ATATGGTAAAGTTACAAGAAGTCATTATTTTAATTGATCAAGTAGTTT**c**agct-5′

Annealing antisense primers. The primers were annealed by heating 20 µL each of the forward and reverse primer stocks in a heat block at 95°C for 2 minutes. The primer mixture was then slowly cooled in the heat block until it reached room temperature. The annealed primers were stored at -20°C for long-term storage.

NcoI and XhoI digestion of pHN678. Restriction enzyme double digestion was performed on pHN678 with NcoI and XhoI according to the manufacturer's protocol (New England BioLabs, Cat. #R0193 and Cat. #R0146 respectively) (14). PCR purification of the cut vector was performed using the PureLink PCR Purification Kit (Cat. #K310001).

Ligation of antisense into pHN678. Ligation was performed using the T4 DNA Ligase manufacturer's protocol for sticky end ligation (15). An insert to plasmid ratio of 30:1 was used. The antisense ligation product was then split into 2 conditions. One condition was left untreated, while the other was digested again with NcoI and XhoI, and then heat inactivated at 80°C for 20 minutes. The plasmid ligated with the annealed antisense primer insert was titled pMOD-wbbL(a), to reflect the modifications made to the antisense design by Chow *et al.*, originally named pCODA-wbbL(a).

Transformation of pMOD-wbbL(a) into TOP10 competent cells. The protocol for this transformation was adapted from the One Shot™ TOP 10 competent cell procedure (16). The ligation product was centrifuged briefly, and then pipetted into microfuge tubes containing TOP10 competent cells. After mixing gently, the tubes were incubated on ice for 30 minutes. The tubes were then transferred to a 42°C water bath for 30 seconds before being placed back on ice. The cells were then transferred from the microfuge tubes into test tubes containing 250 µL of S.O.C medium and incubated at 37°C for 1 hour in a 225 rpm shaker. Cells were then plated on LB medium supplemented with 25 µg/mL chloramphenicol and incubated overnight at 37°C.

RESULTS

Production of pMOD-wbbL(a) as an inducible asRNA silencing model of *wbbL*. The pCODA-wbbL(a) plasmid design generated by Chow *et al.* was modified and used to produce the new plasmid, pMOD-wbbL(a) (Figure 1A) (11). The RNA product of the antisense

primers ligated into pHN678 to generate pMOD-wbbL(a) is designed to be complementary to the ribosome-binding site of the *wbbL* mRNA, targeting a region approximately 50 bps upstream of the mRNA start codon (11, 17). Moreover, the pHN678 plasmid selected for ligation of the antisense primers contains a *lac* operator sequence allowing for induced expression of the asRNA product via Isopropyl β- d-1-thiogalactopyranoside (IPTG), a molecular mimic of allolactose that can trigger *lac* operon transcription (17, 18). This vector also contains NcoI and XhoI restriction enzyme cleavage sites that can be used for ligation of the antisense primers. Many of these features already existed in the original design by Chow *et al.* however, recreation of the construct was necessary because of the lack of availability of pCODA-wbbL(a). Alongside the regeneration of the previous features contained in pCODA-wbbL(a), the primer inserts present in pMOD-wbbL(a) are designed with NcoI and XhoI overhangs and modified so that, following ligation, they would not allow for the regeneration of the NcoI and XhoI cut sites (Figure 1B). This work is done to help streamline downstream screening procedures, a feature which will be further covered in the discussion.

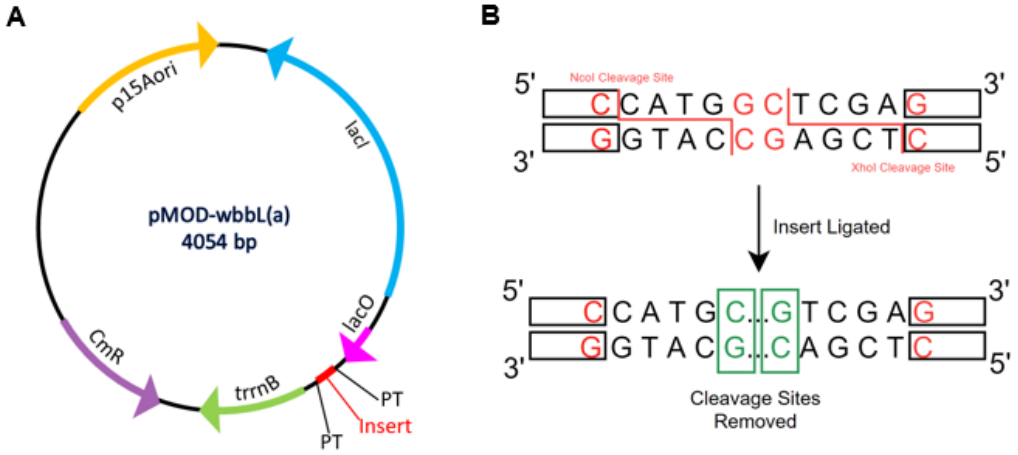

FIG. 1 Overview of pMOD-wbbL(a) plasmid map and cleavage site removal process with insertion of antisense primers. (A) Plasmid map of pMOD-wbbL(a) with *trrnB* encoding a transcription terminator, *CmR* encoding chloramphenicol-resistance, *p15Aori* encoding an origin of replication, *lacI* encoding the lac operon repressor, *lacO* encoding the lac operon, PT denoting paired termini, and insert denoting our designed asRNA sequence. **(B)** Illustration of how the antisense primer designs allow for destruction of NcoI and XhoI cut sites upon ligation into the pHN678 vector, leading to generation of pMOD-wbbL(a).

Transformation of competent MG1655 and DFB1655 L9 with pHN678. We were able to transform both our strain of interest, DFB1655 L9, and the O antigen negative control strain, MG1655, with our empty vector pHN678. This confirms the ability of both strains to uptake pHN678, the chosen plasmid vector in our antisense model design.

Preliminary screening of pMOD-wbbL(a) suggests ligation of antisense primers. Following the ligation protocol, pMOD-wbbL(a) had to be screened to confirm the presence of the *wbbL* antisense primers. The design of the primers destroyed the NcoI and XhoI restriction sites upon ligation, so restriction enzyme analysis was used to screen for inserts. For this process, 10 colonies transformed with pMOD-wbbL(a) were inoculated into LB broth supplemented with 25 µg/mL chloramphenicol and grown at 37°C overnight, with the plasmid isolated and purified the next day. Each plasmid was digested with XhoI digestion (14). The digest products were run on a 1% agarose gel alongside both cut and uncut plasmid controls to compare the ligated plasmids against. The screening results show that XhoI did not cut any of the ten pMOD-wbbL(a) samples, with these lanes appearing much more similar to the uncut control rather than the cut (Figure 2). These results signify that the antisense primers may have ligated into the cut vector and in doing so, prevented the regeneration of

the NcoI and XhoI cleavage sites within the plasmid. Thus, the restriction enzymes would no longer be able to digest the plasmid, with the lanes appearing similar to the uncut control and not the cut.

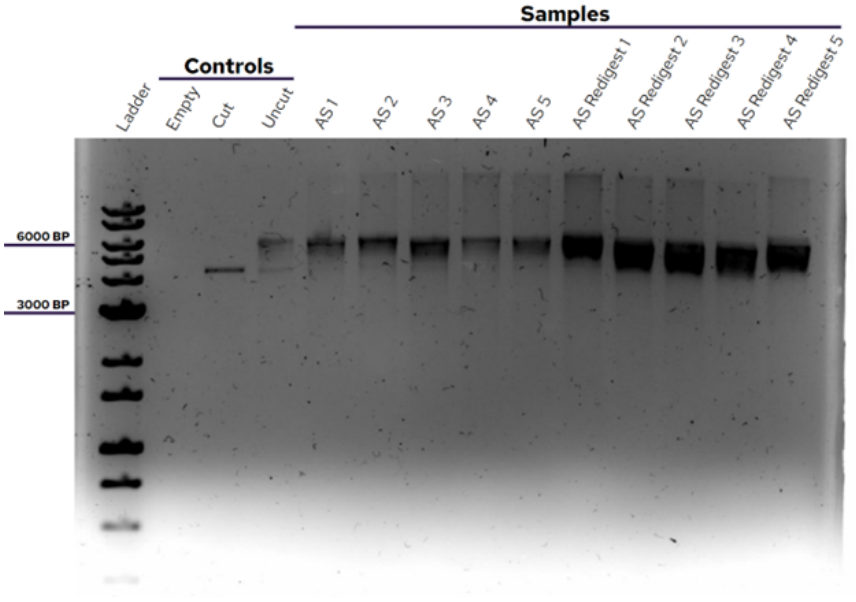

FIG. 2 Screening suggests presence of insert. 1% agarose gel run for 1 hour and 40 mins at 100 V. 1kb+ ladder in first lane supplied by Froggabio (Cat# DM015-R500). The uncut and cut controls provide reference points to determine if insert is present in samples. Samples with insert expected to remain uncut. The numbers (AS 1-5 and AS Redigest 1-5) indicate plasmids isolated from different colonies. All samples, including both AS (asRNA) and AS Redigest (asRNA redigested) appear uncut suggesting presence of insert.

Sequencing of pMOD-wbbL(a) returns inconclusive results. Following the gel screening protocol, six plasmid samples screened were selected for further analysis using Sanger sequencing. Two primers were designed, a forward and a reverse, targeting areas approximately 80 bps upstream and downstream of the insertion site respectively (Table 2).

TABLE. 2 pHN678 primers designed to sequence insert. Primers designed to flank 80 bps upstream and downstream of the insertion site on pHN678. Designed using SnapGene™ (13).

Forward Primer: 5' - CGGCTCGTATAATGTGTGGAATTGT - 3'
Reverse Primer: 5' - ATCAGGCTGAAAATCTTCTCTCATC - 3'

To control for the potential of secondary structure formation, we requested the usage of the Genewiz alternative sequencing protocol for difficult templates (19). Three of the forward reactions, as well as two of the reverse were unable to prime, leaving three forward reactions and four reverse, all of which were considered poor quality. Due to the low signal strength of the reads acquired, it could not be determined whether the insert was present in any of the primed samples. Of note, one of the reverse reactions containing intact NcoI and XhoI cut sites seemed to show a foreign sequence 703 bps long present in-between these cleavage sites. This finding was unexpected, given that the pHN678 plasmid map denoted that these restriction sites should be found next to each other, with no sequences in-between them. These findings point not only to the difficulty of sequencing the insert, but also to the importance of sequencing the empty vector prior to use. The potential discrepancy between the plasmid map and its sequence merits further investigation to understand whether this was a sequencing error, or if a foreign insert does in fact exist between the NcoI and XhoI cleavage sites.

DISCUSSION

The asRNA is designed to bind to the *wbbL* RBS and silence expression at the level of translation. Protein translation is disrupted by asRNAs through either binding to coding mRNAs leading to degradation of the dsRNA hybrid, or occupation of the ribosomal binding site (RBS) (20). Antisense RNA molecules are a subset of noncoding RNA that are often short transcripts consisting of 15-50 nucleotides (9). The complementary nature of the bases that makeup asRNA in relation to its target mRNA is what promotes binding between the two

strands of RNA (20). The stability of asRNA is critical to allow for the oligonucleotide to act on its intended target before degradation by RNase (20, 21). To increase RNA stability, our antisense model modified from Chow *et al.* is designed with paired termini (PT), shown to enhance both stability and gene silencing (22). PTasRNAs contain flanking dsRNA sequences which form a hairpin structure with the asRNA at the tip, intended to stabilize the antisense construct (11, 17, 22). Therefore, because the design of these gene expression regulators is paramount to their function, it is important that we ensure that the sequence is complementary and the structure is stable.

Figure 3 outlines the process by which pMOD-wbbL(a) is designed to interfere with *wbbL* transcription, reducing O antigen expression and potentially T4 bacteriophage resistance. Due to the existence of a *lac* operator sequence upstream of the insertion site, expression of the asRNA can be induced by IPTG. Following expression, the asRNA can diffuse in the cytoplasm and bind to the RBS of *wbbL* as that gene is expressed, thereby inhibiting translation of the mRNA.

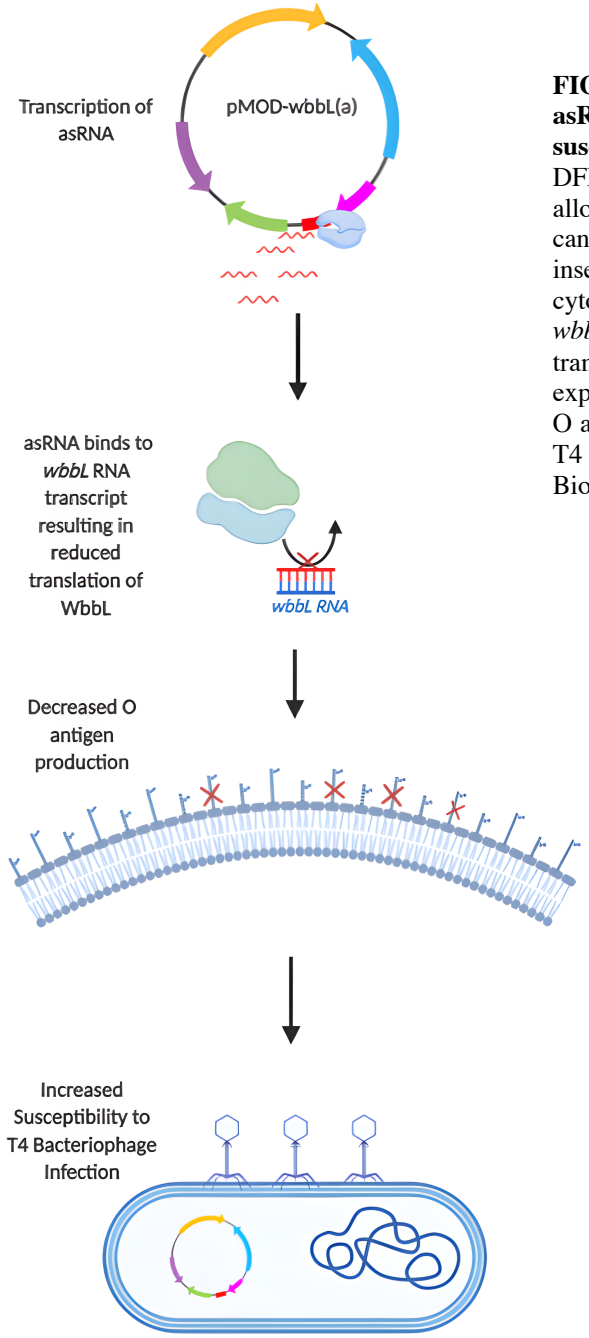

FIG. 3 Overview of the proposed pMOD-wbbL(a) asRNA model in silencing *wbbL* and increasing susceptibility to T4 infection. Transforming *E. coli* DFB1655 L9 cells with pMOD-wbbL(a) would allow for the transcription of our asRNA insert which can be induced using the *lac* operator upstream of our insert and IPTG. The asRNA can then diffuse into the cytoplasm and bind to the ribosome binding site of *wbbL* mRNA transcripts, preventing the mRNA's translation and expression within cells. Reduced expression of WbbL is thought to result in decreased O antigen expression and increased susceptibility to T4 bacteriophage infection. Figure made in BioRender.

A redesigned plasmid was necessary for a more streamlined screening process. We faced significant challenges with Sanger sequencing of our antisense construct pMOD-wbbL(a), with results showing mostly poor quality reads with low signal strengths. The insertion site in pHN678, as touched on above, is designed with PT in order to increase the stability of the RNA product of the antisense insert (22). These PT regions are high in GC content to strengthen pairing interactions as well as decrease probability of RNase degradation of the RNA product (22). However, high GC regions in DNA can often increase chances of secondary structure formation, which can make Sanger sequencing of such areas a strenuous process (23, 24). It has been shown that the polymerase often moves at a slower pace through regions with higher secondary structure elements, the insertion site of the antisense primers being an example of one such region (23). Moreover, Chow *et al.* faced similar challenges in the sequencing pipeline of pCODA-wbbL(a) (11). These known sequencing difficulties, paired with a lack of availability of pCODA-wbbL(a), led to the design of our proposed antisense model, allowing for the screening of ligated plasmids through re-digestion with restriction enzymes and the use of a DNA gel. As outlined previously, our antisense primers work to prevent regeneration of the NcoI and XhoI cleavage sites flanking the insertion location (Figure 1B). Hence, if a ligated plasmid, pMOD-wbbL(a), was to be treated with NcoI, XhoI, or both, these enzymes would not be able to bind and cut the plasmid again. Paired with a pHN678 control, which contains an NcoI and XhoI cut site, one could then observe whether the newly generated plasmid is in fact being cut, and hence does not contain the antisense primers, or appears fully intact on the gel, and hence contains the insert. For instance, while our sequencing results were inconclusive, our gel screening protocol seems to point to the presence of the insert, as none of the screened samples were cut by the restriction enzyme. This introduces a screening step that could help to focus sequencing efforts on a select few plasmid samples that appear uncut following re-digestion. In theory, and with enough time, this alternative design should allow for a more streamlined ligation and screening process.

Downstream analysis is needed to fully understand *the role of wbbL* in T4 Resistance. Although the Chow *et al.* asRNA model did not appear to have a significant effect on the susceptibility of DFB1655 L9 to T4 bacteriophage, they did not have the opportunity to thoroughly test their model (11). We did not generate a plasmid to test this either. Hence, we don't know whether asRNA targeting wbbL will affect phage resistance. It is possible that wbbL targeting asRNA will not result in a decrease in phage resistance. This could be confirmed by measuring O antigen in DFB1655 L9 before and after induction of the asRNA using silver stain sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). If O antigen levels do not vary significantly between the two DFB1655 L9 conditions, this would point to potential issues with the asRNA design or implementation, rather than confirming the lack of involvement of O antigen in T4 bacteriophage resistance.

Limitations Limitations of our study include the lack of a designed or ligated directionality control that would be necessary in downstream testing of our asRNA model to isolate for the effect of induction versus our pCODA-wbbL specifically. Additional limitations of our study include how pMOD-wbbL(a) has only been tested in Top10 Competent *E. coli* thus transformation into the strain of interest DFB1655L9 remains to be explored. Finally, a major limitation of our study is the lack of conclusive sequencing data to confirm the presence of our insert.

Conclusions We adapted the asRNA silencing model developed by Chow *et al.* to create pMOD-wbbL(a) and found through gel electrophoresis screening that XhoI did not cut any of our pMOD-wbbL(a) samples, suggesting the ligation of our asRNA insert. However, Sanger sequencing returned inconclusive results, so it is currently uncertain if our plasmid contains the insert.

Future Directions Proper sequencing of pMOD-wbbL(a) must first be carried out before any future experimental procedures. One way of doing so would be with the addition of DMSO in the sequencing protocol (25). It appears that bulk of the sequencing difficulties arise from the creation of secondary structures inherent to the design of PTasRNAs (19, 22-24). If

sequencing attempts with the addition of DMSO are still unable to proceed properly, the utilization of a different plasmid vector without PT regions may be necessary. Additionally, given the unexpected sequence in-between the NcoI and XhoI cut sites in one of our sequenced samples, sequencing of pHN678 would be a prudent step to ensure the vector is functioning as designed.

If the asRNA sequence within pMOD-wbbL(a) is confirmed to be present, future studies could test whether our asRNA can silence *wbbL* in *E. coli* DFB1655 L9 by measuring WbbL production. Double-overlay phage assays could be completed to see if induction of our asRNA results in decreased T4 bacteriophage resistance. As well, a silver stain SDS PAGE could be performed to see if induction of this asRNA results in lower O antigen concentrations within DFB1655 L9, with MG1655 as a control.

Finally, future studies could also look into designing asRNAs that target other genes in the *rfb* gene cluster, such as *wbbJ, wbbK,* and *wbbI* (1). Like *wbbL*, these genes are also a part of the O antigen biosynthesis operon with their own functions in O antigen production (1). It would be interesting to see how the effects of silencing *wbbL* on T4 bacteriophage and O antigen production compares to the effects of silencing a different gene in the *rfb* gene cluster.

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CONTRIBUTIONS

A.K. Contributed to paper abstract, introduction, materials, methods, and future directions. Helped with digestion, ligation, competent cell preparation, transformation, screening and troubleshooting in the lab; B.N. Contributed to paper discussion, limitations, and figures. Helped with ligation, competent cell preparation, transformation, screening, sequencing preparation and troubleshooting in the lab; G.R. Contributed to paper abstract, materials, methods, figures, future directions, and conclusion. Helped with digestion, ligation, competent cell preparation, transformation, screening and troubleshooting in the lab; A.S. Contributed to paper materials, methods, results, figures, discussion, and future directions. Helped with antisense design, digestion, ligation, transformation, and screening protocols, as well as sequencing preparation and overall troubleshooting in the laboratory. All authors were equally involved in editing of the manuscript.

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