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Antisense RNA Targeting the First Periplasmic Domain of YidC in *Escherichia coli* Appears to Induce Filamentation but Does Not Affect Cell Viability

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YidC is an essential membrane protein in *Escherichia coli*. It plays a crucial role in the proper insertion and localization of many membrane proteins. We aimed to design a vector which expresses an antisense RNA (asRNA) against *yid*C mRNA, which could prevent wild-type *yid*C expression. In further research, this vector can be co-transformed with a construct carrying *yid*C bearing mutations within the periplasmic region in order to study the role of this region of the protein. In this paper, we constructed plasmids which contain an antisense sequence targeting the segment of *yid*C mRNA corresponding to the region encoding the first YidC periplasmic region to prevent YidC translation. The region targeted was downstream of the ribosomal binding site. Two different lengths of antisense inserts, 50 base pairs (bp) and 248 base pairs (bp), were ligated into pHN678 vector, which contains an isopropyl-beta-thiogalactopyranoside (IPTG)-inducible promoter. We successfully generated pNEARH50, pNEARH2481, and pNEARH2482 which were transformed into *Escherichia coli* DH5α. Sequencing results showed that the fragments were inserted in the correct orientation. Plating and growth curve analysis did not show significant differences between the IPTG-induced and uninduced samples. However, the microscopic observations showed filamentous morphology in IPTG-induced DH5α containing the pNEARH50 or pNEARH2482 constructs. This suggests that asRNA expression may have resulted in a partial YidC knockdown phenotype. The partial YidC knockout might be due to the asRNA targeting a sequence downstream to the ribosomal binding site, which is less efficient in preventing translation. This partial knockdown may result in reduced cell division and a filamentous phenotype. We propose that the biogenesis of membrane proteins potentially important for cell division, such as FtsQ, may have been impacted by YidC knockdown.

Membrane proteins are responsible for carrying out a number of critical functions for cell survival (1). Some examples of such functions are energy conversion in photosynthetic complexes, acting as membrane receptors, and membrane transporters (1). The YidC protein is essential for protein insertion in the bacterial membrane in *Escherichia coli* (2) and is highly conserved among pathogenic bacteria (3). In *E. coli*, the YidC protein crosses the membrane six times, having a 319-residue periplasmic domain after the first transmembrane domain (2).

YidC has been shown to be essential for cell viability using the YidC-depletion strain, JS7131. JS7131 was transformed with an araBAD plasmid containing a wild-type *yid*C gene under the control of an arabinose promoter (4). After transformation, JS7131 was grown in Luria-Bertani (LB) broth with either arabinose or glucose (4). With arabinose, the cells were growing at reduced rate, but with glucose, the cells exhibited no growth after 2 days (4). Additionally, cells grown in arabinose which were then diluted into glucose-supplemented media before being grown for several generations exhibited a significant growth defect (4). These results demonstrate the necessity of *yid*C is an essential gene and plays a crucial role in cell survival (4).

The first periplasmic region of YidC makes up more than half of the protein, however a deletion of most of this region was shown to not have an effect on cell growth or on the processing and membrane insertion of proteins (1). The C-terminal region of the periplasmic domain is essential for the membrane insertase function of YidC, as a knockout of the entire periplasmic domain resulted in non-viable cells (5). This C-terminal region has been suggested to play a role in membrane interactions or with binding partners (5). Previous attempts to knockout the function of YidC have been targeted by deletion of certain regions of YidC such as the large periplasmic region (1, 5). To study the function of the first periplasmic domain, we set out to create a vector to inhibit wild-type *yid*C expression. This vector can be used in conjunction with plasmids containing mutations in the *yid*C periplasmic region. A previous study successfully inhibited *yid*C expression with an antisense RNA (asRNA) targeting the ribosomal binding site (3); however, in this experiment we intend to inhibit *yid*C expression with an asRNA targeting the periplasmic domain.

asRNA is a single-stranded RNA molecule which is complementary to the mRNA of a certain gene transcribed in a cell (6). They can be introduced via a plasmid to inhibit translation of a complementary mRNA on a ribosome by forming extensive base pairs with the corresponding mRNA and preventing ribosomal binding and translation (6). asRNA is used by bacteria for gene regulation, especially in some cases where the mRNA with repressed translation encodes proteins which are toxic to the cell in high quantities (6).

We selected the vector pHN678 as it possesses several features that make it optimal for use as an antisense construct. This plasmid establishes a paired-termini design for asRNA as shown in Figure 2 (7). This stabilizes the asRNA by strengthening the base pairing and helps to resist degradation by dsRNA-specific RNAses (7). The increased stability allows the antisense molecule to accumulate after

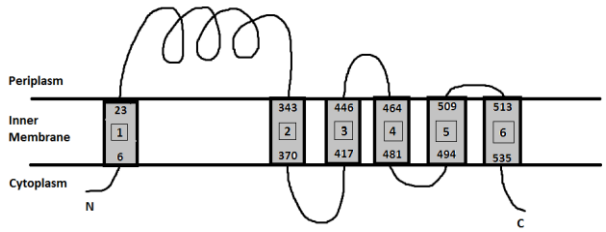

FIG 1 YidC protein structure in the inner membrane. YidC contains a large periplasmic domain between the 1st and 2nd transmembrane domain.

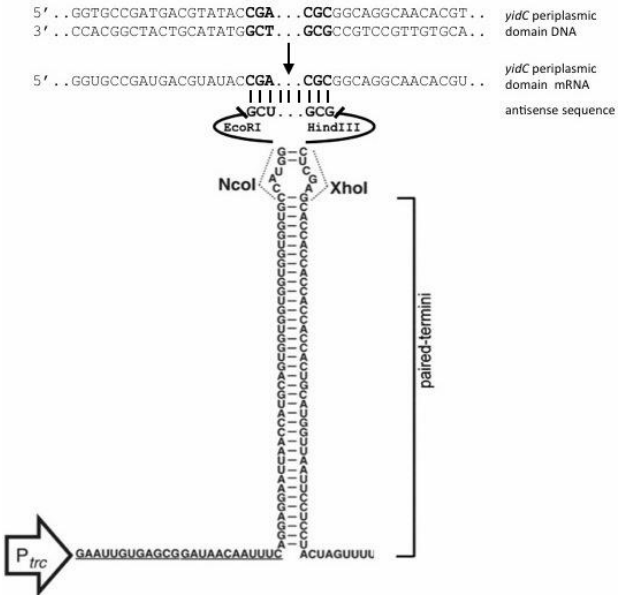

FIG 2 Structure of antisense RNA targeted to the periplasmic *yid*C mRNA.

the plasmid is induced, thus amplifying the inhibition of the target gene (7). Furthermore, pHN678 contains a *lac* operator sequence that allows for IPTG-inducible control (7). In comparison to pBAD24, the promoter is stronger and may increase asRNA expression (7). Thus, pHN678 was chosen as our lead anti-sense expression plasmid, as pBAD24 can create a mixed culture with subpopulations that are differentially induced as a consequence of aspects of arabinose uptake (7).

We hypothesized that asRNA against the periplasmic domain of YidC would knockdown expression of the wild-type YidC protein, resulting in reduced cell viability. To knockdown YidC expression, we used the asRNA strategy targeting the *yid*C mRNA. We inserted 50 bp and 248 bp antisense sequences into pHN678 and created three plasmids, pNEARH50, pNEARH2481, and pNEARH2482. Although the inserts were present and ligated in the correct orientation, we did not observe the YidC knockout phenotype in either the growth curve or the plate growth data following IPTG induction. However, IPTG-induction resulted in a filamentous cell morphology in *E. coli* DH5α transformed with either pNEARH50 or pNEARH2482.

MATERIALS AND METHODS

Strains and growth conditions. *Escherichia coli* DH5α was obtained from the Ramey culture collection (Department of Microbiology and Immunology, University of British Columbia). General growth of *E. coli* DH5α was carried out in LB broth (1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl, cells grown on plates used 1.5% w/v agarose). 30 ug/mL chloramphenicol was added to select for cells transformed with pHN678 or pZ. All growth was carried out at 37˚C, with plates incubated overnight for 18 hours and broths incubated overnight on a shaker at 190 rpm.

Generation of competent cells. 5 mL of overnight *E. coli* DH5α was inoculated into 150mL LB broth (1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl) and grown on a shaker at 37˚C until an OD_{600} between 0.2 and 0.4 was achieved. The cells were pelleted at 4˚C and 10000 rpm for 5 min in a Beckman Coulter JA-20 Fixed Angle rotor, resuspended in 75 mL of 4˚C 0.1M $CaCl_2$, incubated on ice for 40 min, and centrifuged again for 5 min at 10000 rpm. The pellet was then resuspended in 3 mL of 0.1M $CaCl_2$ and incubated on ice for 20 hours before 255 µL aliquot of each cell suspension was mixed with 45 µL of sterile 100% glycerol and frozen at -80˚C until use.

PCR primers design. Primers for the pHN678 vector, summarized in Table 1, were chosen using PrimerQuest (http://www.idtdna.com/Primerquest) with forward and reverse primers being selected from a *yid*C template sequence on *E. coli* MG1655 obtained from EcoCyc (www.ecocyc.org). It has been suggested by previous study that an antisense sequence should have small length, and should have minimal secondary structures (3). Thus, the fragments of *yid*C sequence chosen within the periplasmic domain for antisense expression consisted of nucleotides in the region from +638 to +883 (248 nt) as this was found to produce a minimal amount secondary structures as predicted by OligoAnalyzer 3.1 (http://www.idtdna.com/calc/analyzer) (FIG S1). A set of primers were also designed to generate a 50 bp oligomer for antisense expression against the periplasmic domain of *yid*C from +440 to +490 (FIG S1). Restriction endonuclease sequences were added to the antisense *yid*C primers to ensure that the insert would ligate into the vector in the opposite orientation to generate an antisense RNA in transformed cells.

TABLE 1 Primer sequences (and features) used in the construction of the antisense sequences.

Primer	Restriction enzyme	Primer sequence* (5' TO 3')	Product size (base pairs)
yidC-f-248 (forward *yidC*)	*Hin*dIII	*gata*agctt*CTTCGCACT GCACACCTTCC*	248
yidC-back (reverse *yidC*)	*Eco*RI	*agc*gaattcCCAGTACC GGCTGAGATTTAT AG	
EcoR1-b-50 (forward oligomer)	*Hin*dIII	agctGCAGGTGCCGA TGACGTATACCGA CGCGGCAGGCAAC ACGTTTACCAAA	50
Hind3-f-50 (Reverse oligomer)	*Eco*RI	aattTTTTGGTAAAC GTGTTGCCTGCCG CGTCGGTATACGT CATCGGCACCTGC	

*Upper case nucleotides are complementary to sequence; lower case nucleotides are restriction enzyme sequence; italicized nucleotides are additional nucleotides added to complete restriction enzyme site to PCR products.

Plasmid isolation from DH5α. Plasmid pHN678 and pZ were provided by Dr. Liam Good (Royal Veterinary College, University of London) in precipitate form, and were resuspended in autoclaved distilled water and then transformed to DH5α competent cells via heat shock transformation. Isolated DH5α colonies containing pHN678 or pZ were grown in a 5 mL overnight culture, with the appropriate antibiotic, on a shaker at 37ºC. 1 mL of the culture was used for plasmid isolation using the PureLink Quick Plasmid DNA Miniprep Kits (Invitrogen, K2100) according to the manufacturer's protocol.

Polymerase chain reaction, agarose gel electrophoresis, and insert purification. 35 cycles of PCR amplification was carried out in a Bio-Rad Gene Cycler™ thermocycler with the following protocol: denaturation at 94 ºC for 15s, annealing at 55 ºC for 30s, and extension at 68 ºC for 25s. For amplification of *yid*C periplasm antisense inserts, a PCR reaction was set up with 1 µL of isolated pEH1YidC-GFP (Dr. Joen Luirink, Vrije Universiteit), 1.5 µL of each 10uM forward and reverse primers, 5 µL of 10X *Pfx* Amplification Buffer (Invitrogen, 11708-013), 1 µL of 50 mM $MgSO_4$, 1.5 µL of 10 mM dNTP mix, 0.4 µL of Platinum® *Pfx* DNA Polymerase (Invitrogen, 11708-013), and 38.1 µL of dH_2O. 15 µL of PCR reactions were mixed with 3 µL of 6X loading buffer (Fermentas, R0611), and 15 µL of sample was loaded on to a 2% (w/v) agarose gel for electrophoresis. Gels were run in 1X TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.5) at 90 V for 50 minutes. The gels were stained in 0.5 ug/mL of ethidium bromide for 20 minutes prior to visualization on the Alpha Innotech AlphaImager. DNA purification was carried out using PureLink™ PCR Purification Kit (Invitrogen, K3100-01) according to the manufacturer's instructions.

Oligonucleotide generation and phosphorylation. EcoR1-b-50 and Hind3-f-50 were annealed according to the Protocol for Annealing Oligonucleotides (Sigma-Aldrich). Annealing buffer (10 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA) was first added to 34 µL of dH_2O, which 10 µL of the mixture was added later to 45 µL of each primer during the resuspension step.
Oligonucleotide phosphorylation reaction was set up with 30 µL of oligonucleotide, 5 µL of 10X T4PNK Reaction Buffer (NEB, B0201S), 5 µL of 10 mM ATP, T4 PNK (NEB, M0201S), and 9 µL of dH_2O.

Antisense periplasmic *yid*C cloning. Isolated pHN678 and 248 bp antisense *yid*C insert restriction digestion reactions were set up as follows: 10 µL of plasmid or purified PCR inserts, 1 µL *Eco*RI (NEB, R0101S), 1 µL *Hin*dIII-HF (NEB, R3104S), 5 µL 10X NEBuffer 2.1 (NEB, B7202S), and 33 µL dH_2O. Reaction mixes were incubated at 37 ºC for 65 min followed by heat inactivation at 80 ºC for 20 min.

The ligation reaction protocol was adapted from Ligation Protocol with T4 DNA Ligase (NEB, M0202). Reactions that contain digested pHN678, digested 248 bp antisense *yid*C inserts or phosphorylated 50 bp inserts, T4 DNA ligase buffer (Invitrogen, 15224-041), and T4 DNA Ligase (Invitrogen, 15224-041) were incubated at room temperature for 10 minutes. Ligation reactions with pZ and antisense inserts were also performed as positive control.

Transformation into competent cells. 5 µL of ligation reaction mixtures were added to 50 µL of DH5α competent cells and incubated on ice for 30 min. Heat shock was carried out at 42˚C for 30 sec. Mixture was then immediately transferred into 950 µL of LB broth, and culture was incubated in a 37˚C for 1 hr to recover. 100 µL of the cells were then plated onto LB agar plates containing 30 µg/mL chloramphenicol for pHN678 or pZ containing transformants, and incubated at 37˚C overnight.
Colony PCR was carried out to confirm transformation of pHN678 with 248 bp antisense *yid*C inserts. Restriction digest with *Eco*RI (NEB, R0101S) and gel electrophoresis were carried out to confirm transformation of pHN678 with 50 bp antisense *yid*C inserts.
Positive colonies were used to inoculate overnight cultures in LB broth containing 30 µg/mL chloramphenicol. Plasmids were isolated using the PureLink Quick Plasmid DNA Miniprep Kits (Invitrogen, K2100) according to the manufacturer's protocol.

Sequencing. Plasmid from 2 mL of overnight culture for each sample was isolated using the PureLink Quick Plasmid DNA Miniprep Kits (Invitrogen, K2100). Autoclaved water was substituted for TE. 10 µL of 24.1 µg/ml pNEARH50, 34.2 µg/ml pNEARH2481, or 34.4 µg/ml pNEARH2482 were sent out for sequencing at GENEWIZ with 5 µL of Hind3-f-50r.

Growth of IPTG-induced and uninduced DH5α on LB plates. DH5α wild-type (WT), DH5α carrying pZ, pNEARH50, pNEARH2481, or pNEARH2482 were cultured in 5 mL of LB broth with 30 µg/mL chloramphenicol on a shaker at 37ºC. The overnight culture was diluted 10000X in fresh LB, and 5 µL of each sample was spotted onto LB agar plates with 30 µg/mL chloramphenicol. The plates were either induced with 20 mM isopropyl-beta-thiogalactopyranoside (IPTG) (Fermentas, R0392) or uninduced. The plates were incubated at 37ºC for 15 hours and growth was compared.

Growth curve of IPTG-induced or uninduced DH5α in LB broth. To assess cell growth via inhibition of *yid*C, *E. coli* DH5α (WT, pZ, pNEARH50, pNEARH2481, or pNEARH2482) were cultured overnight in 50 mL of LB with 30 µg/mL of chloramphenicol on a shaker at 37ºC. After 16 hours, 1 mL of overnight culture was transferred into 50 mL of fresh LB with 30 µg/mL of chloramphenicol. Upon reaching OD_{600} of approximately 0.2, each culture was split into 2 samples of 25 mL each, one of which was induced with 1 mM IPTG (Fermentas, R0392). For the first hour, OD_{600} readings were monitored with Ultrospec 3000 UV/Visible Spectrophotometer every 30 minutes. After 60 minutes, OD_{600} readings were recorded every hour until late log phase was reached.

Microscopic observation of IPTG-induced and uninduced DH5α. To visualize cell growth, a loopful of each sample was taken from the growth curve experiment during exponential growth phase, 300 minutes after experiment started. Samples were visualized via Kyowa Unilux-12 Microscope at 1000x magnification with oil immersion. Images were obtained using AxioVision.

RESULTS

Sequencing of pNEARH50, pNEARH2481 and pNEAR2482 confirms the success of the antisense plasmid construction. To confirm the sequence of the constructed pHN678 vectors with the antisense inserts, we selected 1 colony with the 50 bp insert and 2 colonies with 248 bp insert for sequencing. The plasmid names and features are summarized in Table 2. GENEWIZ sequencing results indicated successful ligation of inserts into pHN678. We compared the sequence of pNEARH2481 and pNEARH2482 to the pHN678 vector sequence map (FIG S6, FIG S7). The 248 bp inserts were present and ligated in the inverted position, which gives rise to a non-coding complementary strand which can bind *yid*C mRNA (comparison data in supplementary section). Since the Hind3-f-50r primer was used to sequence plasmids carrying the 50 bp inserts, the presence of a sequenced product indicated successful insertion of the fragment (FIG S8). Analysis of downstream sequences indicated correct direction of ligation.

TABLE 2 Summary of *E. coli* strain and cloning vectors used.

***E. coli* K23 strain**	**Genotype**
DH5α	F-; *deoR*; *endA1*; *gyrA96;hsdR17(rk-mk+); recA1; relA1; supE44; thi-1; Δ(lacZYA- argFV169); f80lacZ; DM15*

Cloning Vectors	**Features**
pEH1YidC-GFP	*yidC*
pHN687	IPTG inducible promoter (P_{trc}), paired-termini flanking MCS, Cam^R
pZ	pHN678 derivative, IPTG-inducible promoter (P_{trc}), Cam^R, *ftsZ* antisense insert
pNEARH50	pHN678-a*yidC***, 50bp insert
pNEARH2481/pNEARH2482*	pHN678-a*yidC***, 248bp insert

*denotes duplicated samples with the same insert
**denotes antisense *yidC* insert

IPTG-induced DH5α harbouring antisense constructs exhibit minimal YidC knockdown in comparison to uninduced DH5α harbouring antisense constructs. A 1/10000 dilution of DH5α harbouring pHN678 (WT), pZ, pNEARH50, pNEARH2481, or pNEARH2482 was plated for each sample. As shown in Figure 3, uninduced pZ exhibited normal growth but IPTG-induced pZ exhibited no growth. IPTG-induced WT exhibited less growth than uninduced WT, which indicates that IPTG may reduce bacterial viability. The colonies of DH5α with pNEARH50, pNEARH2481, and pNEARH2482 were also less dense on the induced plate than uninduced plate. In comparison to the WT control, this difference is insignificant and may be independent of the presence of the asRNA plasmids.

No significant differences were observed between IPTG-induced and uninduced DH5α harbouring pNEARH50, pNEARH2481, pNEARH2482. Optical density at 600 nm was used to monitor DH5α growth and determine the inhibitory effect of *yid*C antisense expression. Figure 4 compares the growth of either IPTG-induced or uninduced DH5α harbouring pHN678 (WT), pZ, pNEARH50, pNEARH2481, or pNEARH2482. For WT, OD_{600} of uninduced DH5α exceeded OD_{600} of IPTG-induced DH5α at 350 minutes, which indicates the presence of IPTG decreased the growth rate of DH5α near the end of the log phase. At the endpoint (452 minutes), this difference reached 0.18 OD units. For pZ, similar growth was observed between induced and uninduced DH5α until 80 minutes, after which uninduced DH5α showed consistently higher growth rate than induced DH5α. At the endpoint, the difference between induced and uninduced DH5α reached 0.723 OD units.

The endpoint OD_{600} difference between IPTG-uninduced and induced DH5α was 0.282 OD units for pNEARH50, 0.201 OD units for pNEARH2481 and 0.131 OD units for pNEAR2482. Compared to the 0.18 OD units difference in the WT control, the difference in DH5α expressing the asRNA is insignificant.

pNEARH2481 and pNEARH2482 exhibited slightly different growth curves despite being identical in sequence. This may result from natural variation in biological systems, and questions how significant the observed results are. Duplicating each sample will allow statistical analysis of the significance of the observed difference.

Hence, under the conditions tested, no significant differences were observed between the IPTG-induced and uninduced cultures except in DH5α harbouring pZ. The growth of *E. coli* DH5a was not significantly affected by the expression of *yid*C asRNA complementary to the periplasmic region of YidC.

Induction of antisense constructs yields a filamentous morphology. Microscope images in Figure 5 reveal the distinct morphology of uninduced and IPTG-induced DH5α. Both the uninduced and induced WT had rod-shape morphology and similar length to width ratio. This indicates bacterial morphology is not affected by IPTG. Uninduced pZ was rod-shaped but induced pZ was filamentous, with the length to width ratio exceeding 50:1. This confirmed the potency of the IPTG used.

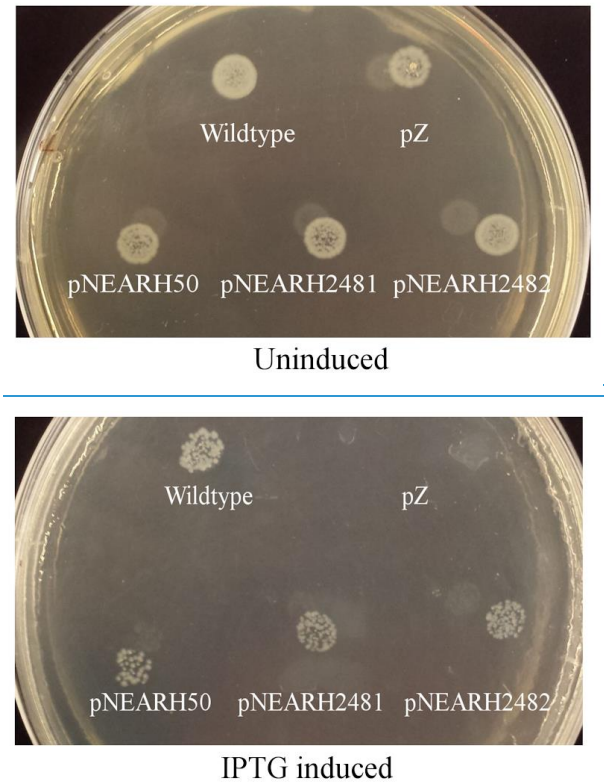

FIG 3. Comparison of growth of *E. coli* DH5α on LB plates either induced with 20 mM IPTG or uninduced. Only pZ exhibited significantly decreased viability after IPTG induction. The wild-type, pNEARH50, pNEARH2481, pNEARH2482 colonies did not demonstrate significant IPTG-induced reduction in growth.

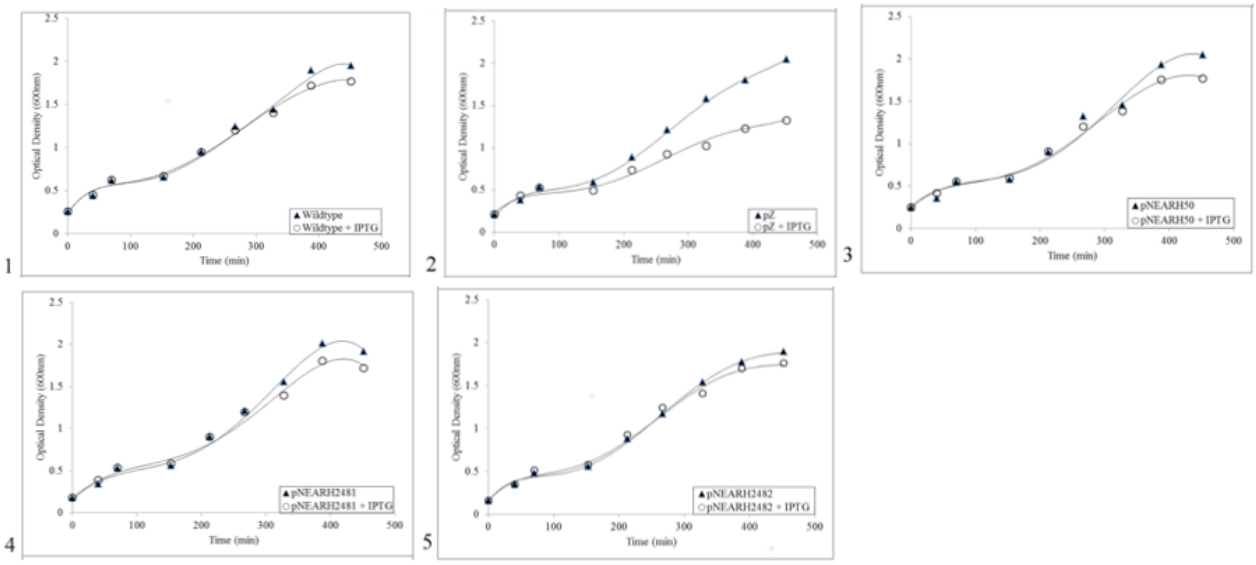

FIG 4. **The effect of IPTG on the growth rate of wild-type (containing pHN678 without the antisense insert) DH5α and DH5α transformed with pZ, pNEARH50, pNEARH2481, and pNEARH2482 plasmids.** Only pZ exhibited a significant decrease in growth after IPTG induction. pNEARH50, pNEARH2481, and pNEARH2482 exhibited an insignificant decrease in growth. IPTG-induction appears to reduce the growth rate of all cultures. IPTG was added at 0 mins.

The uninduced cells containing pNEARH50, pNEARH2481 and pNEARH2482 exhibited a rod-shaped morphology comparable to wild-type. In contrast, induced pNEARH50 and pNEARH2482 showed filamentation in some bacteria, although the length to width ratio was significantly less than pZ. This indicates that the asRNA may have a modest effect on YidC expression resulting in a partial knockdown. The partial knockdown does not affect bacterial viability (Figure 3) and growth rate (Figure 4) but alters bacterial morphology.

DISCUSSION

Three plasmids, pNEARH50, pNEARH2481, pNEARH2482, were created and they encode asRNA complementary to the *yid*C periplasmic region. However, the attempt to generate a YidC knockout phenotype was unsuccessful. In generating the asRNA, 2 approaches were attempted. We designed primers against the *yid*C periplasmic region downstream of the RBS to generate a 248 bp amplicon. We also designed a synthetic 50 bp oligonucleotide corresponding to the periplasmic region. Both inserts were separately ligated into pHN678 in an inverted position to allow for expression of the non-coding strand. It was expected that asRNA expression would prevent YidC expression and decrease cell viability.

The effect of the antisense constructs was evaluated via plate growth, growth curve, and microscopic observation of cell morphology. All three protocols utilized two controls: wild type (WT) *E. coli* DH5α transformed with either pHN678 or pZ. WT *E. coli* DH5α harbours the pHN678 without the antisense insert and was used as a standard for comparison with DH5α bearing antisense constructs. pZ was used as the second control because it allows for the IPTG inducible expression of *ftsZ* asRNA (8). FtsZ is highly conserved among bacteria and is a prokaryotic homologue of eukaryotic tubulin which assembles into a contractile ring (called the Z-ring) at the mid-cell site during cell division (8). FtsZ assembly into the Z-ring is essential to cell division, and inhibition of FtsZ leads to the inability to divide, contributing to filamentous cell morphology and increased cell death (8).

IPTG itself is unlikely to have been responsible for the results displayed in Figures 3 and 4. As shown in Figure 3, all IPTG-induced DH5α exhibited lower density of growth than uninduced DH5α. This includes the WT control, which is less dense on IPTG-induced plates than uninduced plates. Moreover, in Figure 4, the growth of all IPTG-induced DH5α, including the WT control, was less than uninduced DH5α. The could due to the presence of IPTG decreasing cell growth and viability unrelated to the presence of asRNA expression, as there have been previous experiments demonstrating that IPTG has a negative effect on cell growth (9). However, Figure 5 shows that bacterial morphology of uninduced and IPTG-induced WT cells were similar, while IPTG-induced DH5α carrying antisense constructs demonstrated filamentation when induced. Therefore, it is unclear whether IPTG alone was responsible for the differences observed in the growth curve, and replicates would be necessary in order to draw a conclusion.

As shown in Figure 5, with respect to pNEARH50 and

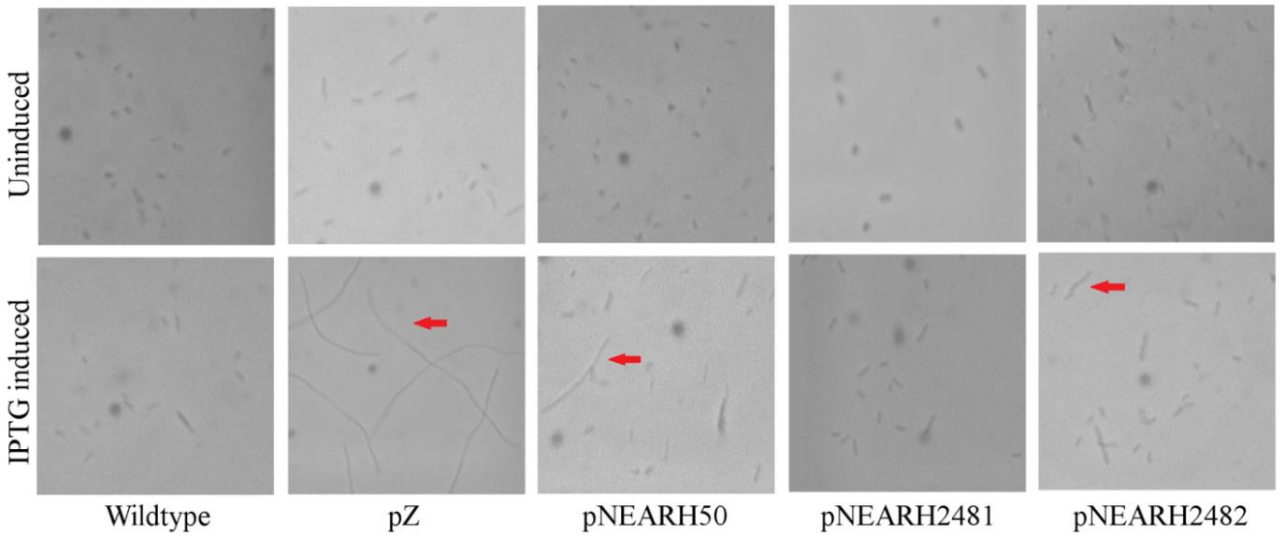

FIG 5. **IPTG induction altered cell morphology in *E.coli* DH5α with pZ, pNEARH50, pNEARH2481, and pNEARH2482.** IPTG-induced pZ exhibited a filamentous phenotype due to *fts*Z asRNA expression, which inhibits cell division. Some IPTG-induced pNEARH2482 and pNEARH50 exhibited an elongated phenotype, indicating a partial *yid*C knockout potentially affecting membrane proteins important in cell division.

pNEARH2482 transformed cells, a percentage of the bacteria demonstrated an elongated morphology as well as an increased length to width ratio, indicating an altered phenotype. Hence, the asRNA expression may be inhibiting the translation of a fraction of all *yid*C mRNA transcripts, but the difference is not significant enough to be reflected in decreased cell viability and growth as shown in Figure 4.

YidC is required by many different membrane proteins for proper membrane localization and insertion (10). One protein which is a substrate to YidC is the cell division protein, FtsQ. FtsQ an essential protein during cell division in *E. coli* since it interacts with many other Fts proteins (11). A partial knockout of YidC could prevent the proper localization or membrane insertion of FtsQ which could lead to the inhibition of cell division leading to a filamentous morphology of bacterial cells.

The cells were able to survive despite induction of the antisense construct. A possible explanation for this is that we targeted the middle of the mRNA, whereas previous experiments have successfully targeted the ribosome binding site (12), Therefore, the lack of YidC knockout phenotype may indicate that an antisense fragment targeted to a region downstream of the mRNA RBS may be less efficient in inhibiting translation. Dryselius *et al.* examined antisense inhibition of beta-lactamase gene and *acp*P and demonstrated that peptide nucleic acids (PNA) can inhibit bacterial gene expression with gene sequence specificity (12). The study illustrated that the start codon including the shine-dalgarno sequence is most sensitive to inhibition and most reliable site to target asRNAs. This site of ribosome assembly and translation is dependent on the recognition of this sequence, hence, this site is susceptible to steric interference. The study also demonstrated that asRNAs targeted outside this region were ineffective.

Our results did not demonstrate the inhibition of cell growth. This result is consistent with the observation that ribosomes are difficult to disassemble once the translation process has started (13). For our experiments, since the ribosome has already associated and is translating when it comes across the complementary base-paired sequence, it might knock the asRNA off of the *yid*C mRNA. Hence, there may only be a partial inhibition of translation.

In summary, the antisense plasmids pNEARH50, pNEARH2481, and pNEARH2482 were successfully constructed to investigate whether an antisense RNA strategy targeting *yid*C mRNA sequences downstream of the ribosomal binding site would lead to cell death. Induction of these antisense constructs appears to alter the bacterial morphology, however the desired effect of total YidC knockout leading to cell death is not observed. Therefore, further investigation is required into what caused the morphology change as well as to what degree asRNA was expressed when the plasmid was induced under the conditions tested. In conclusion, targeting mRNA sequences against the periplasmic region downstream of the ribosomal binding site may cause partial knockdown of genomic *yid*C expression.

FUTURE DIRECTIONS

Features that contribute to asRNA success include short-length, minimal secondary structures, and targeting the ribosomal binding site (rbs). Previous research with asRNA against the rbs induced cell death with complete YidC knockdown (3). One problem is that targeting the

periplasmic region is novel, and we did not have a plasmid to compare and confirm our observations. We propose generating an asRNA targeting YidC rbs, which should completely knockdown of *yid*C expression. pNEARH50, pNEARH2841, and pNEARH2842 can be compared with the positive control to confirm the partial knockdown phenotype observed.

It is also worth noting that the pBAD24-derived constructs we created were not tested. The pBAD24 vector contains an araBAD (arabinose) operon with regulatory gene *araC*. Future work should test the pBAD24-derived constructs in an ara$^-$ *E. coli* strain, using growth curve, plate growth, and microscopy to observe the effect of YidC knockdown.

Finally, an experiment can test the pHN678-derived constructs using IPTG disks of various concentrations. Using an overnight culture of pNEARH50, pNEARH2841, or pNEARH2842, inoculate an agar plate and incubate with disks of different IPTG concentrations. After a confluent lawn can be observed, examine the areas near IPTG disks, which should display clearance from asRNA induced cell death. This approach is less labour-intensive than the growth curve. It is also uses a smaller amount of IPTG, with the benefit of reduced cost in comparison to growth curve and plate growth experiments. This experiment would investigate the effects of increasing asRNA concentration on cell viability.

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