Plasmid Mediated Complementation of YidC Using a YidC Variant Fused to Green Fluorescence Protein Supports Growth of *Escherichia coli* BL21 at 30^{O}C but not at 37^{O}C

(Note: Erratum associated with this manuscript.)

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ABSTRACT

ERRATUM: Follow up studies of the strain described in this study have shown that they are *Rhizobium* and not *E. coli* (Hooshmand *et al*, JEMI, Volume 21, pages 101 – 106**). The described biological characteristics of these strains are therefore not valid. Nevertheless, the approach designed to build these strains may be of interest to future researchers.** YidC is an essential membrane protein in Gram-negative bacteria involved in translocation and folding of proteins into the inner membrane. Previous studies have shown that the depletion of YidC disrupts folding of penicillin binding proteins leading to defects in peptidoglycan biosynthesis and cell division. YidC is a 60 kDa inner membrane protein with 6 transmembrane domains and a 30 kDa soluble domain (termed P1) located between transmembrane domains 1 and 2 that extends into the periplasm. P1 is not essential for cell viability as mutant strains of *Escherichia coli* expressing in frame deletions of P1 are viable. The function of P1is unknown. Given the relatively large size of P1 and its evolutionary conservation in Gram-negative bacteria, it is likely to have an important biological function. In this experiment, we designed a chromosomal *yidC* knock out strain of *Escherichia coli* by complementing expression witha YidC variant fused at its C-terminus to green fluorescence protein encoded on a plasmid. We compared the phenotype of an *Escherichia coli* strain expressing a P1 deleted form of YidC with a strain expressing a wildtype copy of YidC. Our preliminary observations suggest that the strain expression of the P1 deleted YidC protein forms rod-shaped cells similar to the wild type strain. These data suggest that the P1 domain is not involved in maintaining cell shape. Interestingly, the knockout strain of *Escherichia coli* expressing YidC fused to GFP grow at 30^{o}C but not at 37^{o}C suggesting a temperature sensitive phenotype, which may be useful when constructing YidC mutants in future structure-function studies.

INTRODUCTION

Membrane-embedded proteins perform a variety of functions in bacteria. YidC is a 60 kDa essential protein in Gram-negative bacteria that is composed of 548 amino acids. It is a component of the protein translocation machinery in the inner membrane that is involved in membrane protein insertion and Sec co-regulated chaperone activities that involves the translocation of polypeptides across the membrane (3). YidC spans the plasma membrane in 6 transmembrane (TM) domains with a ~35kDa periplasmic domain 1 (P1) positioned between transmembrane domains 1 and 2. The P1 domain makes up more than of half of the full-length protein. The crystal structure of YidC has been characterized. It comprises five TM α-helices (TM2–6) and with protruding P1 and C-terminus domain regions. The P1 domain is connected to the TM region by the PH1 helix, which is amphipathic and lies parallel to the plane of the membrane. (2, 3, 5). The function of YidC P1 domain is not well understood, yet its relatively large proportion in YidC and its evolutionary conservation suggests that it may play an important role in the cell (2, 3, 5). YidC P1 domain is not required for cell viability, since plasmids expressing YidC lacking the P1 domain are able to support cell growth (2). It is therefore possible to perform in frame deletion experiments to study its function.

YidC has been demonstrated to predominantly localize at the poles of the plasma membrane using YidC-green fluorescent fusion protein (YidC-GFP) (12). Xu *et al* tested attempted to test whether or not the YidC P1 domain is able to affect the polarization of YidC in the cell membrane using a variant of YidC-GFP bearing an in frame deletion of the P1 domain (11). In these studies, strains of *Escherichia coli* expressing wild type YidC and mutant YidC bearing an inframe deletion of the P1 domain were expressed from plasmids, while the chromosomal copy of YidC remained unaltered. We sought to follow up this study by constructing strains bearing deletion of the chromosomal copy of YidC that were complemented with the YidC expression plasmids previously described by Xu *et al* (11).

YidC is involved in the insertion of membrane proteins and folding of periplasmic proteins, such as the penicillin binding proteins (PBP). Misfolding or incorrect insertion of these membrane proteins could result defects in cell division or slower growth rates (1, 7, 9). However, the mechanism by which YidC facilitates protein-folding in the periplasm remains unclear. That YidC-mediated folding activities are carried out in the periplasm suggests that the P1

domain could have an important role in this in this process. Incorrect folding or insertion of membrane and periplasmic proteins could lead to defects in cell division (15). In this study we aimed to investigate the function of the YidC P1 domain. We hypothesized that an in frame deletion of the YidC P1 domain would induce elongated cell shape. To test this hypothesis we constructed a chromosomal deletion of the *yidC* gene in E. coli strain BL21. YidC expression was complemented with a plasmid expressing YidC fused at its C-terminus to GFP. In one variant the P1 domain had been deleted and the other expressed a wild type (WT) full length YidC.

Our data suggest that YidC P1 is not required for maintaining cell shape. Interestingly, we were able to observe growth of the YidC-GFP complemented strains of *E. coli* at 30ºC but not at 37ºC, suggesting of a temperature sensitive phenotype.

MATERIALS AND METHODS

Bacterial stains and growth conditions. Bacterial strains used for this study were obtained from the Microbiology & Immunology department at the University of British Columbia and are listed in Supplemental Table 1 in supplemental materials. *E.coli* BL21(DE3) was used as the recipient strain for all the plasmids. *E.coli* strains were grown in Lysogeny Broth (LB) medium at 37ºC at 200 rpm, except the strain that contains temperature-sensitive pKD46 plasmid which was grown at 30ºC at 200rpm. Solid LB medium contained 1.5% agar. Appropriate antibiotics were added at the following final concentrations: 100 µg/ml ampicillin (amp), 50 µg/ml kanamycin (kan), and 25 µg/ml chloramphenicol (cam).

Confirmation of P1 deletion via sequencing. To validate the YidC P1 domain deletion performed by the Xu *et al* (11), DNA extractions were conducted to obtain pEH1-YidC-GFP and pYidC-Δp1-GFP-Xu plasmids using ThermoFisher PureLink Quick Plasmid Miniprep Kit. The plasmids were sent for sequencing to the UBC NAPS facility. Refer to Table 2 in supplemental materials for primer sequences.

Preparation of chemically competent *E.coli* BL21. Strains containing pEH1-YidC-GFP and pYidC-Δp1-GFP- obtained from Xu *et al*. were inoculated in 3 mL LB broth in test tubes and grown at 37ºC at 200 rpm. After 16 hours of growth, the subcultures were used to inoculate 150 mL LB broth in 500 mL Erlenmeyer flasks. The cultures were incubated at 37ºC at 200 rpm to achieve the OD_{600} of 0.55. The cells were then harvested using centrifugation and resuspended in Inoue Transformation Buffer to gain competency (14).

Construction of chromosomal deletion. BL21 cells containing either pEH1-YidC-GFP or pYidC-Δp1-GFP-Xu were made chemically competent and transformed with pKD46 plasmid that encodes recombinase (10). 500 ng of the pKD46 plasmid was added to 100 µl of competent cells, mixed gently by swirling, and incubated on ice for 30 minutes. The mixture was incubated at 42ºC in a water bath for 90 seconds to facilitate uptake of pKD46 . The tube was immediately placed on ice for two minutes and 900 µl of SOC medium was added. The mixture was incubated at 30ºC for an hour with agitation. 100 µl of the culture was used for spread plating and the LB plates were incubated at 30ºC overnight.

Chloramphenicol cassette construction. PCR amplification was performed to construct the chloramphenicol cassette with the *yidC*-specific flanking regions. Primers for the PCR reaction were designed to have segments of DNA that are homologous to the upstream and downstream of *yidC* as well as segments to amplify the chloramphenicol resistance gene from the pKD3 vector. The culture of, the *E.coli* strain BW25141 containing pKD3 was grown at 37ºC and the plasmid was extracted using a PureLink® Quick Plasmid Miniprep Kit. 120 ng of the extracted pKD3 was used as template for PCR reaction in order to amplify the cassette. Gradient PCR ranging from 50ºC to 60ºC was performed.Amplificationto of the cassette was then validated by performing gel electrophoresis with 1.0% agarose gel.

Confirmation of genomic insertion of *camR*. Genomic insertion of the chloramphenicol cassette was verified by PCR amplification. Colony PCR was performed using the isolated colonies of WJKC-1 containing pEH1-YidC-GFP, pKD46, and the recombined chloramphenicol cassette, and WJKC-2 strain that contains pYidC-Δp1-GFP-Xu, pKD46 and the cassette. Primers designed to bind 300 bp upstream and downstream of *yidC* were used verify the size of the cassette in individual transformants. The optimal annealing temperature for the PCR reaction was 55ºC. Refer to Supplemental Table 2 in supplemental materials for primer sequences.

IPTG-induced growth of *E. coli* strains. After confirming that recombination had occurred, transformants of strains WJKC-1 and WJKC-2 were subjected to varying concentrations of IPTG in the presence of ampicillin, kanamycin, and chloramphenicol. 0.0 mM, 0.5 mM, 2.5 mM, and 10 mM IPTG was used in order to induce the expression of either the wildtype or the mutant YidC from the pEH1-YidC-GFP and pYidC-Δp1-GFP-Xu plasmids (11). The transformants were incubated at 30ºC and the OD_{600} was measured for observing growth.

Temperature-sensitive growth experiments. Transformants after chloramphenicol cassette transformation containing both YidC P1 deletion plasmid and WT plasmid were picked from same colony and streaked onto same plates containing Cam and Kan antibiotic induced with 100 mM IPTG. WJKC-1 and WJKC-2 strains cells were then incubated at room temperature 30^{o}C and 37^{o}C for 48 hrs. Growth was compared by observing colony formation.

Fluorescence microscopy. Both WJKC-1 and WJKC-2 strains were then induced with 100 mM IPTG in Amp Cam and Kan containing Lysogeny broth and grown at 30^{o}C overnight. Fluorescent microscopy was performed, and images were taken. Wet mounts of the bacterial culture were prepared without fixing the cells. 10-20 uL of dense bacteria culture was added to microscope slide and promptly covered with glass cover slip. Fluorescent microscopy was performed using Zeiss Axiostar Plus fluorescence microscope with a 490 nm excitation filter (FITC channel). Pictures were taken using iphone 5 digital through the eye-piece. Images were cropped and processed using ImageJ.

RESULTS

Sequencing of plasmid-containing *yidC*-WT and Δp1 shows deletion of the *yidC* P1 domain. To confirm that pYidC-Δp1-GFP-Xu contained the deletion of the P1 domain (amino acids 23-343), we designed sequencing primers as described in Supplemental Table 2 targeting regions upstream and downstream of the *yid*C P1 domain in both pEH1-YidC-GFP and pYidC-Δp1-GFP-Xu. As shown in Table 2 in supplementary materials, the forward primer targeted the start codon (Met23) of YidC P1 domain, while the reverse primer targeted 100bps (Tyr376) downstream of the same domain. Nucleotide sequence analysis showed a deletion of the P1 domain in pYidC-Δp1-GFP-Xu as shown in Supplemental Table 2. Nucleotides encoding amino acids 23-317 were absent in the plasmid sequence. However, nucleotide coding for amino acids 318 to 343 were observed. Plasmid pEH1-YidC-GFP showed 99% sequence identity to the respective region of the reference *yidC* sequence asdepicted in in Supplemental Table 2. These results suggest that Xu *et al.* had created an in-frame deletion of amino acids 23 to 317 of YidC, which represents the majority of the P1 domain in pYidC-Δp1-GFP-Xu.

Gel electrophoresis demonstrates the construction of the *camR*-cassette. To determine the ideal annealing temperature for the amplification of the *camR*-cassette, DNA gel electrophoresis was performed for the six PCR samples. The presence and intensity of the 1133 bp band, corresponding to the *camR*-cassette, was analyzed. Figure 1 shows distinct bands just above 1000 bp in lanes 1 through 6, consistent with the expected size of the

chloramphenicol resistance gene with the flanking regions. The bands seem to be more prominent in lanes 1, 2 and 3, suggesting that the optimal annealing temperature for the gradient PCR was in the ranges of 56.3°C - 60°C. The bands were gel-extracted and purified. There are faint nonspecific bands present in lanes 1, 2, and 3 between 2000-3000 bp and also just below 200 bp. The no-template negative control did not show any bands. These bands are undesirable as they illustrate impurities in the PCR product. Taken together these data support the conclusion that the *camR* cassette had been amplified.

Construction of chromosomal *yidC* knockout strain. To facilitate lambda-red recombination of chromosomal *yidC* and *camR*-cassette, *E. coli* BL21 pEH1-YidC-GFP and pYidC-Δp1-GFP-Xu were transformed with pKD46 and plated onto LB agar containing ampicillin and kanamycin. Following 24 hours of incubation in 30^{O}C, approximately 150 colonies appeared. A single colony of each of the *E. coli* BL21 transformants was used in electrocompetent cell preparation. Following L-arabinose induction of the recombinase gene on pKD46, both *E. coli* BL21 strains were electroporated with the *camR*-cassette, and plated onto LB agar containing ampicillin, kanamycin, and chloramphenicol. After 96hrs of incubation, approximately 100 colonies appeared, suggesting recombination between chromosomal *yidC* and the cassette. The putative *yid*C knockout strains grew slowly at 30oC suggesting adecrease in growth rate for both WJKC-1 and WJKC-2.

To verify the chromosomal *yidC* deletion, primers were designed to amplify the chromosomal *yidC* region. PCR products were analyzed with gel electrophoresis. Figure 2B shows three of six samples with bands of molecular weights slightly below 1500 bp. These faint bands may represent the *camR* gene, which is around 1400 bp. A very bright band above the 2000 bp marker is clearly visible in the positive control lane 9, representing the chromosomal *yidC* of around 2200 bp. Taken together, these results suggest that the chromosomal copy of *yidC* has been deleted.

***E. coli* BL21 WJKC-1 and WJKC-2 grow at 20^{O}C and 30^{O}C but not at 37^{O}C.** Preliminary experiments were performed to characterize *E. coli* WJKC-1 and WJKC-2. As seen in Figure 6, after incubation on LB agar containing kanamycin and chloramphenicol, the strains appear to grow very slowly or not at all at 37^{O}C but grew well at 30^{O}C and 20^{O}C. These data suggest that these strains may be sensitive to growth at temperatures above 30^{O}C as a result of the chromosomal *yidC* knockout. In addition, growth rate of each strain correlated directly with IPTG concentration (Figure 7), supporting the conclusion that expression of YidC from the plasmid is linked to the observed phenotype. Finally, we looked any morphological changes in cell shape related to the deletion of the P1 domain. As seen in Figure

4 and 5, initial observations under the microscope suggested that both strains fluoresced green, confirming expression of the GFP fusion in both strains. Cell shape appeared similar (rod-shaped), respectively. These results suggest that the P1 domain is not essential for maintaining cell shape.

DISCUSSION

In this study, we have investigated the function of the YidC P1 domain on cell shape and growth rate. Using *E.coli* BL21 DE3, we created two chromosomal *yidC* deletion strains (*E. coli* WJKC-1 and WJKC-2) using the lambda-red recombination system and complemented the strain with plasmid pEH1-YidC-GFP or pYidC-Δp1-GFP-Xu. Our results suggest that chromosomal *yidC* has been knocked out. The YidC P1 domain does not seem to be responsible for maintaining cell shape as bacteria appeared rod shaped under the microscope. While growth rate of each strain appeared to correlate with inducer concentration the mutants strains expression YidC fused to GFP grew at 20oC and 30ºC but not at 37ºC.

As pYidC-Δp1-GFP-Xu had not been verified after construction, we first verified the P1 deletion by sequencing. The sequence of pYidC-Δp1-GFP-Xu, however, revealed that the 25 C-terminal amino acids of the P1 domain are still intact and the deletion spans amino acid residues 23 - 318. Also, we confirmed that even though the deletion is partial, the coding sequence is still in frame and thus the residues downstream of the P1 domain are unaffected. We do not know the function of the remaining 23 amino acids (318 to 343) of P1. They could be important in YidC function, as cells expressing pEH1-YidC-GFP and pYidC-Δp1-GFP-Xu have similar cell shape. It could be that the 25 residues at the C-terminus of P1 are important for the activity of YidC.

The principal contribution of constructing a chromosomal *yidC* deletion strain is to create a platform to express the pYidC-Δp1-GFP-Xu and pEH1-YidC-GFP so that the phenotype of YidC P1 domain deletion can be compared with WT. Our findings suggest that chromosomal *yidC* has been knocked out. First, colonies were able to grow on LB plates containing chloramphenicol after transformation. This indicates the transformants have taken up the *camR*-cassette and that recombination had occurred. Also, PCR did not amplify chromosomal *yidC*, which is expected to be around 2200bp from the *camR* transformants (Figure 2). This finding suggests that the recombination event happened at the correct location and chromosomal *yidC* has been crossed out. The reason that *camR*-cassette could not be amplified clearly in *E. coli* WJKC-1 and WJKC-2 remains uncertain. As shown in Figure 2B, the faint band around

1400 bp indicates that there are not a lot of PCR products for the *camR*-cassette region. We speculated that they could be secondary structure formed on the template that inhibited the function of DNA polymerase and lead to fewer products. Due to the quality of the PCR result, further investigation is needed to confirm the knockout on a genetic level. Future studies could investigate whether adding low concentration of DMSO which break the secondary structure would increase the yield of PCR product.

In addition to our genetic studies, IPTG induced growth of the WJKC strains expressing pEH1-YidC-GFP and pYidC-Δp1-GFP-Xu also suggests that chromosomal *yidC* has been deleted. Cell growth is directly related with increasing concentration of IPTG as shown in Figure 7. Our findings indicate that growths of *E. coli* WJKC-1 and WJKC-2 are regulated by the expression of pEH1-YidC-GFP/pYidC-Δp1-GFP-Xu, which is induced by IPTG. As an essential gene, YidC is required for cell growth, and if the WJKC strains are relying on pEH1-YidC-GFP/pYidC-Δp1-GFP-Xu expression to produce YidC, it suggests that chromosomal *yidC* is deleted and thus it cannot contribute to express YidC, which is independent of IPTG induction. Previous studies have reported similar findings on L-arabinose-induced YidC plasmid (13), where they observed significant growth defects in Δ*yidC* JS7131 cells containing arabinose-induced YidC plasmid but growing with minimal arabinose. However, in our study, the effect of antibiotics on the growth of the transformants is not well characterized. As observed in Figure 7, *E. coli* WJKC-2 treated with antibiotics have achieved maximum growth after 24 hours at different IPTG concentrations.

The morphology of WJKC strains containing pEH1-YidC-GFP/pYidC-Δp1-GFP-Xu has no observable difference under light microscope and fluorescent microscope. Contrary to our hypothesis, our findings indicate that no elongated cell shape is observed in *E. coli* WJKC-2, which suggests YidC P1 domain does not play a role in inducing an elongated cell shape in *E. coli*. Whether or not P1 is involved in other aspects of cell physiology is unknown. Further studies testing binding specificity of YidC P1 with various membrane and periplasmic protein could be done to further investigate the question.

Interestingly the WJKC strains expressing either pEH1-YidC-GFP or pYidC-Δp1-GFP-Xu grow at 20^{o}C and 30^{o}C but not at 37^{o}C. One explanation is that chromosomal insertion had a polar effect on the expression of a downstream *yid*C gene . Until the region of *yid*C disrupted in this study is sequenced extensive about the mechanism unpinning the temperature sensitive phenotype is not warranted.

In conclusion, the preliminary observation suggests that the P1 domain of YidC is not required to maintain cell shape. As noted above, we have yet to confirm chromosomal *yidC* deletion through sequencing. However, the PCR result using WJKC DNA template combined with IPTG induction growth assay suggest that the WJKC strain with chromosomal *yidC* deletion was constructed and its growth was regulated by the pEH1-YidC-GFP or pYidC-Δp1-GFP-Xu expression, which is correlates with increasing concentrations of IPTG. Expression of pEH1-YidC-GFP or pYidC-Δp1-GFP-Xu was confirmed using fluorescence microscopy.. *E. coli* strains WJKC-1 and WJKC-2 showed a temperature-sensitive growth phenotype. The reason for this temperature sensitive growth phenotype is currently unknown.

FUTURE DIRECTIONS

The plasmids used to rescue the expression of *yidC* encode an incomplete deletion of the P1 domain. To test whether the remaining 23 amino acid C-terminal residues (amino acids 318 to 343) do not play a role in the temperature-sensitive growth phenotype or IPTG-induced growth, one could construct a plasmid with a complete P1 deletion and complement the knock-out strain with this plasmid. PCR could be used to amplify the region flanking of the expected recombination site and a region in the cassette in each *yid*C knock out strain. This PCR product can be analysed by gel electrophoresis and sequenced. Finally, it would be interesting to further test the constructed *E. coli* strains for a phenotype related to deletion of the P1 domain. It this regard it would be interesting to measure antibiotic resistance to antibiotics targeting peptidoglycan synthesis using either disk diffusion assays or minimum inhibitory concentration broth dilution assays.

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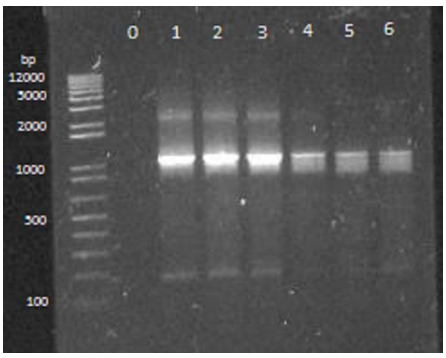
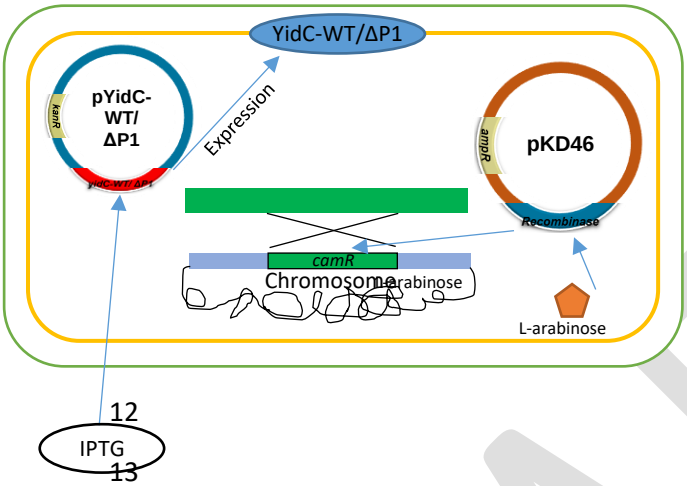
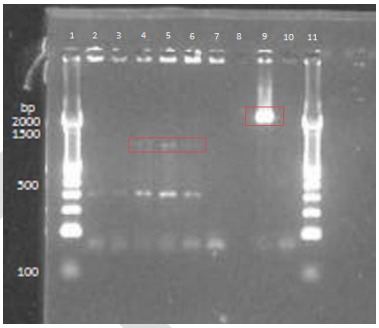


Lane 0: (-) control
Lanes 1-6: Chloramphenicol + flanking region
1 = 60.0 °C
2 = 58.3 °C
3 = 56.3 °C
4 = 53.9 °C
5 = 52.0 °C
6 = 50.0 °C

FIG. 1. Gel Electrophoresis of the Chloramphenicol cassette. Flanking region were designed using gradient PCR with primers as shown in Supplemental Table 1. The products were ran on 1.5% agarose gel at 120V for 30min. Molecular weight of ~1000bp corresponds to the *camR*-cassette (intended PCR product).

A





Lanes 1, 11: Ladder
Lanes 2-4: WJKC-1
Lanes 5-7: WJKC-2
Lane 9: (+) Control
Lane 10: (-) Control

yidC band = 2200 bp
camR band = 1400 bp

FIG. 2. Transformation of pKD46 and *camR*-cassette into BL21 pYidC-WT and pYidC-ΔP1. A) shows induction of pYidC-WT or pYidC -DP1 by IPTG while pKD46 expresses recombinase required for recombination of the camR cassette at *yid*C. Recombinase expression is induced with L-arabinose. B) A gel of the PCR amplified chromosomal *yidC* region for validation of *camR*- cassette recombination.

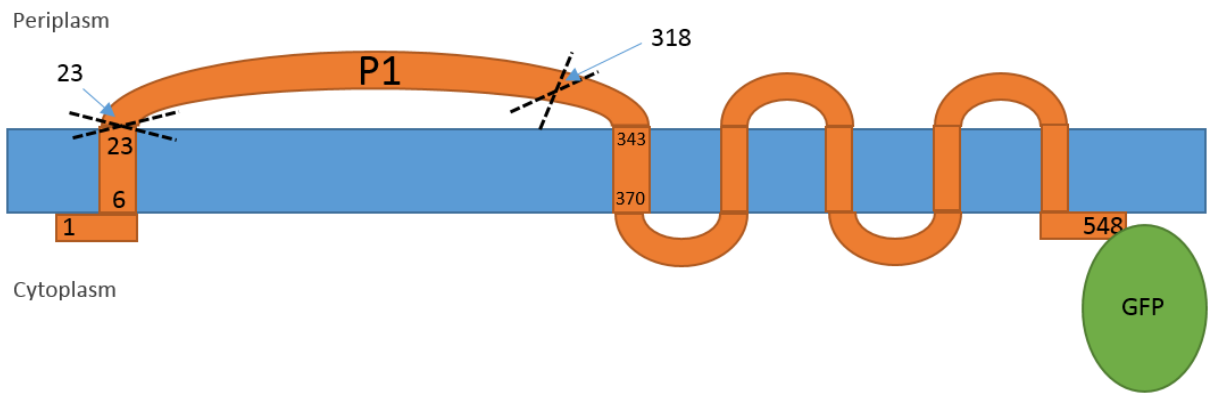


FIG. 3. Amino acid topography of the YidC-GFP transmembrane protein. P1 domain in the YidC protein is 320 amino acids, starting from amino acid 23 to 343. Our sequencing data, as shown in Supplemental Table 2, depicts the P1 knockout between amino acids 23 and 318. The dotted lines depicts where the deletion of P1 occurred. The GFP was induced by IPTG for verification of expression of *yidC*.

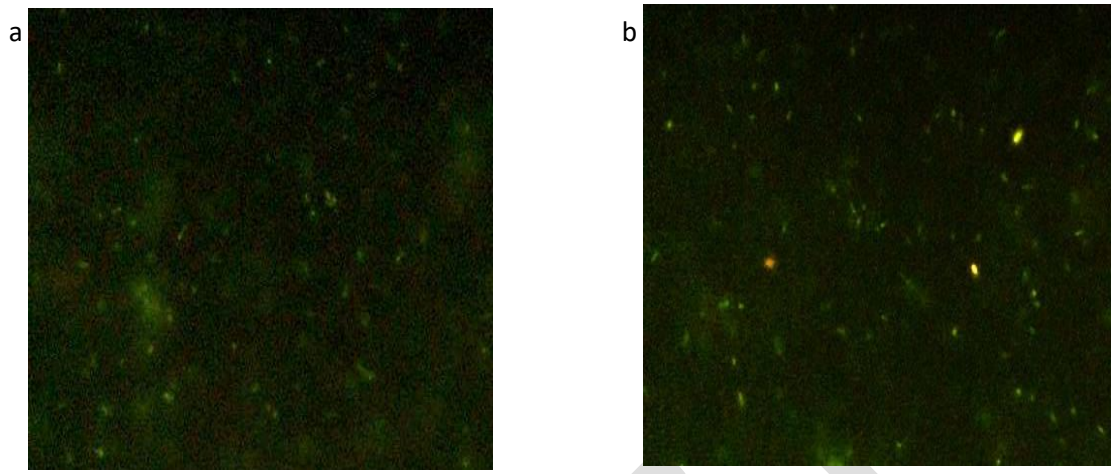


FIG. 4. Induction of GFP in pEH1-YidC-GFP/pYidC-Δp1-GFP-Xu in *E. coli* BL21 using 200mM of IPTG. a) *E. coli* WJKC-1 and b) BL21 WJKC-2 were grown in LB + ampicillin, chloramphenicol, and kanamycin on shaking incubator (30ºC) for 12hrs. When both cultures reached OD_{600} = 0.6, IPTG was added to each culture for a final concentration of 200mM and incubated on shaking incubator (30ºC) for an additional 2hrs. Each culture sample were viewed using fluorescent microscopy (100x).

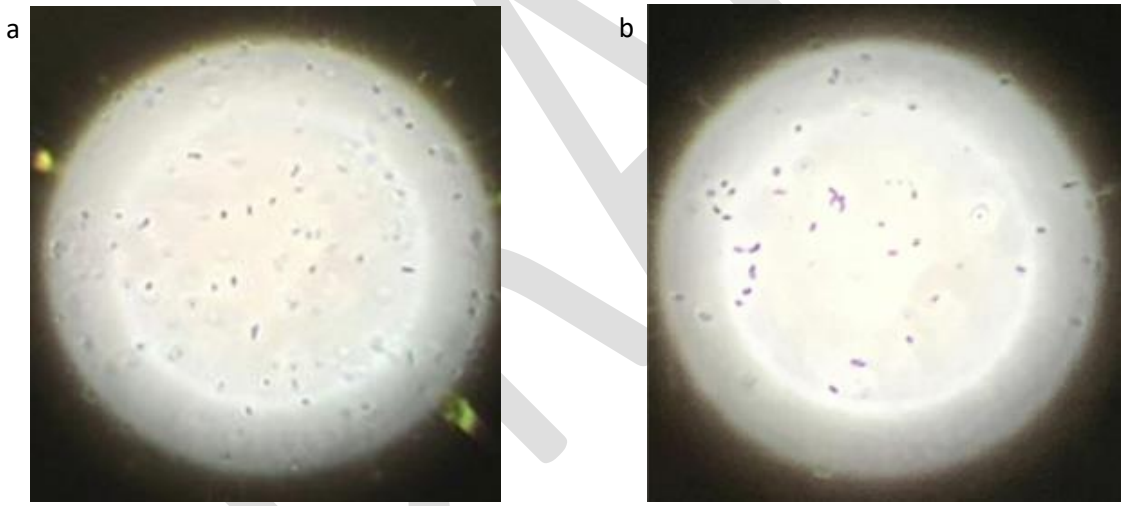


FIG. 5. Comparison of Morphology between BL21 YidC-WT and P1 knockout. a) *E. coli* BL21 pYidC-WT+pKD46+*camR* and b) BL21 pYidC-ΔP1+pKD46+*camR* were grown in LB containing amp, cam , and kan + 100mM IPTG on shaking incubator (30ºC) for 24hrs. Wet mounts of 10uL of each culture sample were viewed using phase contrast microscopy (100x).

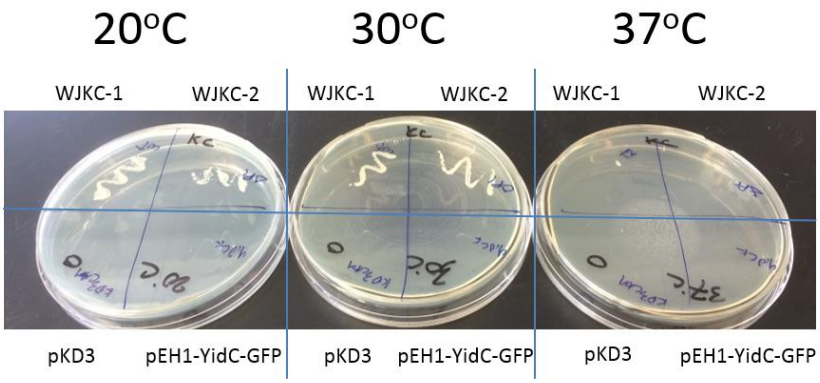


FIG. 6. Temperature sensitivity test of YidC mutants. *E. coli* BL21 YidC P1 knockout and WT were grown in 20ºC, 30ºC, and 37ºC incubators on LB plates containing chloramphenicol + kanamycin + 200mM IPTG for 96 hours. In addition, controls (pKD3 and pEH1-YidC-GFP) were grown in the same conditions. Growth on plates was monitored every 24hrs. YidC mutants incubated at 37ºC did not grow, whereas at 20ºC and 30ºC, clear streak growth was observed.

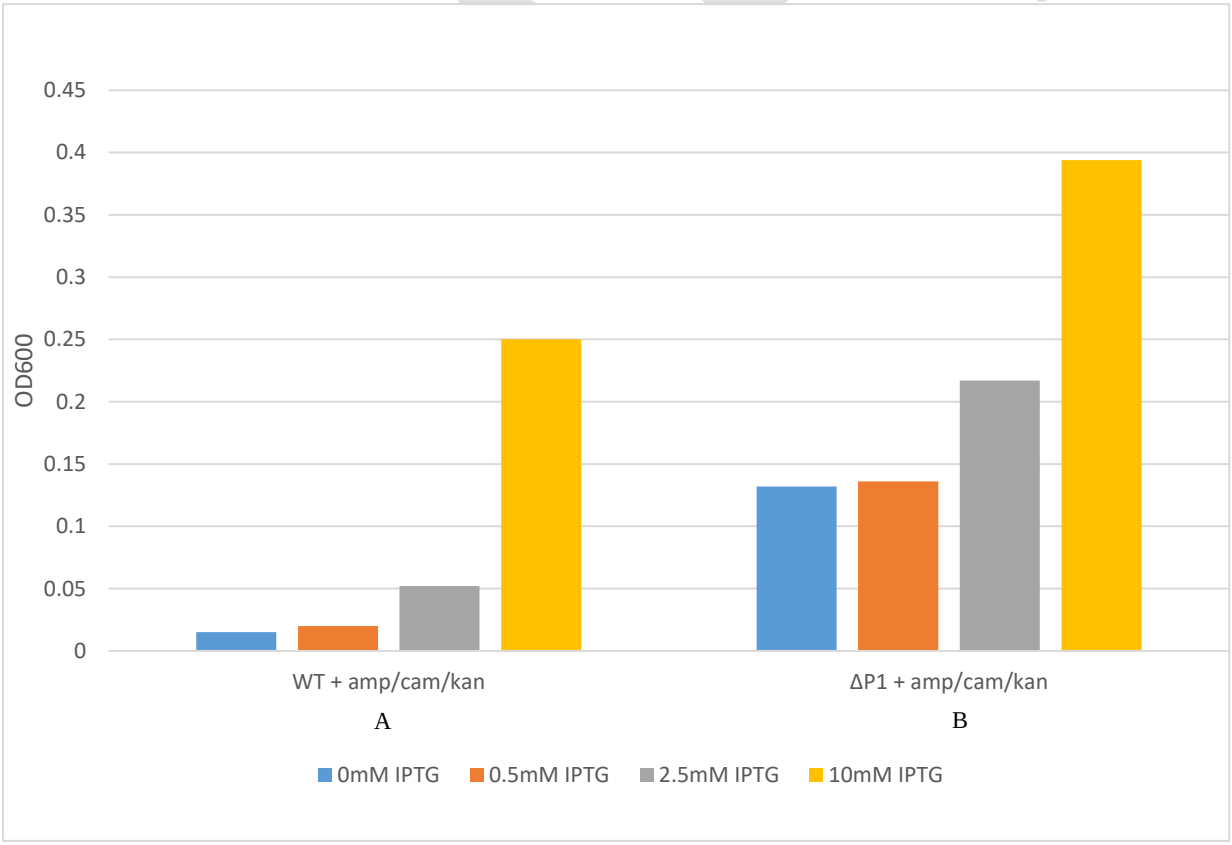


FIG. 7. Induction of *E. coli* BL12 Using Combinations of Different IPTG Concentrations and Antibiotics. A) BL21 pYidC-WT-pKD46-*camR* and B) BL21 pYidC-ΔP1-pKD46-*camR* were grown in LB + ampicillin, chloramphenicol, and kanamycin. Varying amounts of IPTG were also added to each sample to check for induction of pYidC-WT and pYidC-ΔP1. All cultures were grown on 30ºC shaking incubator for 24hrs, and measured for OD_{600} using spectrophotometer.