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The Major Periplasmic Domain of YidC May Be Required for Polar Localization of a Green Fluorescence Protein Tagged YidC Variant Protein in *Escherichia coli*

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Membrane proteins play an essential role in the survival of prokaryotic cells. YidC is a transmembrane protein that functions to insert proteins into the cell membrane either through the Sec translocase dependent pathway or an alternative independent pathway. YidC is 548 amino acids in length, and is comprised of six transmembrane domains and numerous cytoplasmic/periplasmic domains, the biggest being the first periplasmic domain (P1) which is 319 amino acids long. The specific function of YidC P1 is still not well characterized. Previous studies have shown that YidC fused to green fluorescence protein localizes to the cell poles. We hypothesize that the P1 periplasmic region directs the polar localization of YidC. Here we describe a PCR deletion method, using homologous-end designed primers to create a deletion of the P1 domain in a YidC-GFP variant. The resulting nucleotide sequence of the deletion construct was determined to confirm the in-frame deletion in P1 of YidC-GFP. Fluorescence and bright field microscopy were used to observe localization of the mutant YidC-GFP protein. BL21 (DE3) cells expressing either the YidC-GFP P1 deletion construct or the YidC-GFP wild type construct were compared. Our preliminary observations suggest that deletion of the YidC-GFP P1 domain results in circumferential localization of the YidC protein in BL21 cells whereas wild type YidC-GFP was observed at the cell poles. We also conclude the deletion of the P1 region in YidC-GFP does not affect cell viability.

In prokaryotic cells, membrane-embedded proteins perform a variety of essential molecular functions. YidC is a 60 kDa essential inner membrane protein that facilitates and catalyzes the biogenesis, folding and insertion of other membrane protein into the inner membrane in *Escherichia coli* (1). YidC spans the inner membrane six times, and contains a large 35-kDa (319 amino acid) periplasmic domain between transmembrane domains 1 and 2 ($YidC_{EC}P1$) (2). Even though the six transmembrane domains are essential to the survival of the *E. coli* cell, the deletion of the large part of the $YidC_{EC}P1$ domain does not affect cell viability. However, $YidC_{EC}P1$ has been found to

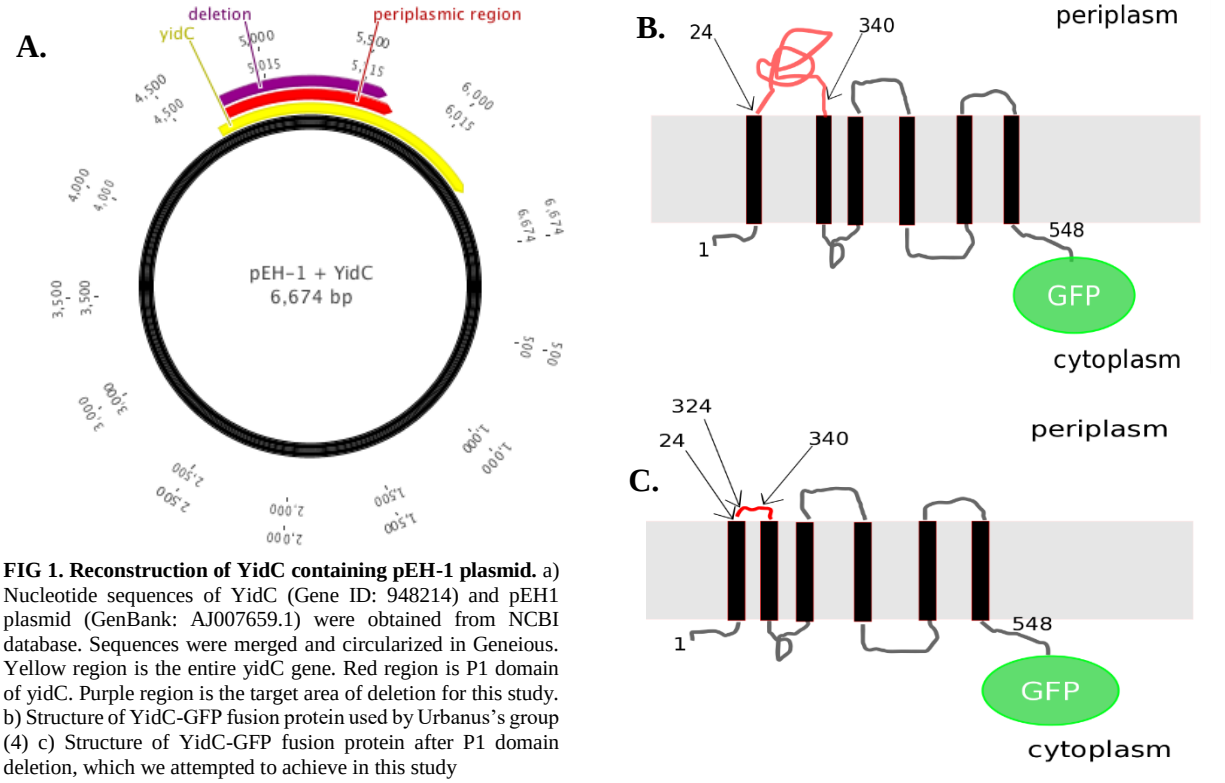


FIG 1. Reconstruction of YidC containing pEH-1 plasmid. a) Nucleotide sequences of YidC (Gene ID: 948214) and pEH1 plasmid (GenBank: AJ007659.1) were obtained from NCBI database. Sequences were merged and circularized in Geneious. Yellow region is the entire yidC gene. Red region is P1 domain of yidC. Purple region is the target area of deletion for this study. b) Structure of YidC-GFP fusion protein used by Urbanus's group (4) c) Structure of YidC-GFP fusion protein after P1 domain deletion, which we attempted to achieve in this study

be evolutionarily conserved in almost all Gram-negative bacteria, leading to the prediction that the $YidC_{EC}P1$ may have important functions. In *E. coli*, YidC has been reported to help localize many other membrane proteins (1).

Previous studies revealed that majority of the large periplasmic domain is not required for YidC function (1). Moreover, YidC lacking amino acid residues 25-323 is still functional (1) and able to support the growth of *E. coli*. Deletion of residues 265-346 resulted in non-functional YidC and failure of bacterial growth (3). To study the distribution of YidC within the plasma membrane, one group constructed a novel YidC-GFP fusion protein by adding a green fluorescence protein to the C terminus of YidC, expressed it in *E. coli*, and observed fluorescence preferentially localized at the polar ends of bacteria, indicating YidC localizes to the cell poles (4). However, the mechanism by which YidC localizes was not determined. Analysis of $YidC_{EC}P1$ domain suggests roles in membrane interaction or potential regulation of YidC with other binding proteins (5). $YidC_{EC}P1$ may be essential in localizing YidC to the two poles of cells. In this study, we explored the role of $YidC_{EC}P1$ in localizing YidC to the poles of cells by deleting $YidC_{EC}P1$ from the YidC-green fluorescence protein (GFP) fusion protein construct made by *Urbanus et al.* (4), shown in figure 1b. We successfully created a YidC-GFP construct bearing a deletion of the $YidC_{EC}P1$ region, structure shown in figure 1c. We show that $YidC_{EC}P1$ may be involved in localizing YidC to the poles in *E. coli*.

MATERIALS AND METHODS

Bacterial Strains and growth conditions. BL21(DE3) and DH5a were obtained from the Microbiology & Immunology department at the University of British Columbia. *E. coli* DH5a was used to amplify plasmid. BL21 (DE3) strain was used to express YidC. Subcloning Efficiency™ DH5α™ (Invitrogen) was used to linearize PCR products. Growth media used was Luria broth +/- agar +/- kanamycin. Bacteria were grown in aerobic conditions at 37 °C. LB medium was prepared in deionized water with 10 g/l NaCl, 10 g/l Tryptone, 5 g/l yeast extract, and adjusted to pH 7. Kanamycin were used at final concentration of 30 µg/ml.

Harvesting and transformation of competent BL21(DE3) and DH5α cells. Bacterial cell cultures were prepared using 3 ml LB broth in a test tube. The culture was grown overnight at 37°C at 200 rpm. The next day, 3 ml of the overnight bacterial cell cultures was used to inoculate 150 ml of LB broth in a 250 ml flask, and the flask was immediately grown at 37 °C at 200 rpm for two hours. Upon reaching an OD_{660} reading of 0.35, the bacterial cells were transferred to sterile, ice-cold 50 polypropylene tubes and cooled to 0°C for 10 minutes. Cells were then recovered by centrifuging at 2700 x g for 10 minutes at 4°C. After the supernatant had been discarded, 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$, 20 mM $CaCl_2$ in deionized water) were added to the tube. Cells were then recovered by centrifuge at 2700 x g for 10 minutes at 4°C. After the supernatant had been discarded, 2 ml of ice-cold 0.1 M $CaCl_2$ solution was added to the tube and re-suspended gently. The competent BL21 bacterial cells were then dispensed into aliquots of 200 µl in 1.5 ml microfuge tubes at -80°C for future use.

Various versions of YidC containing-pEH-1 plasmids were used to transform competent cells. 70 ng of the plasmid was added to 200 µl of competent cells, mixed gently by swirling, and stored on ice for 30 minutes. The tubes containing the mixture of competent cells and plasmid DNA were then transferred to a preheated 42°C water bath for exactly 90 seconds. Then the tube was rapidly transferred to an ice bath and allowed to chill for two minutes. 800 µl of fresh LB media was added to the tube. The tubes were then placed and incubated at 37°C at 200 rpm for one hour. After one hour, 100 µl of the transformed cell culture was plated on LB-kanamycin (30 µg/ml) plates and incubated at 37 °C.

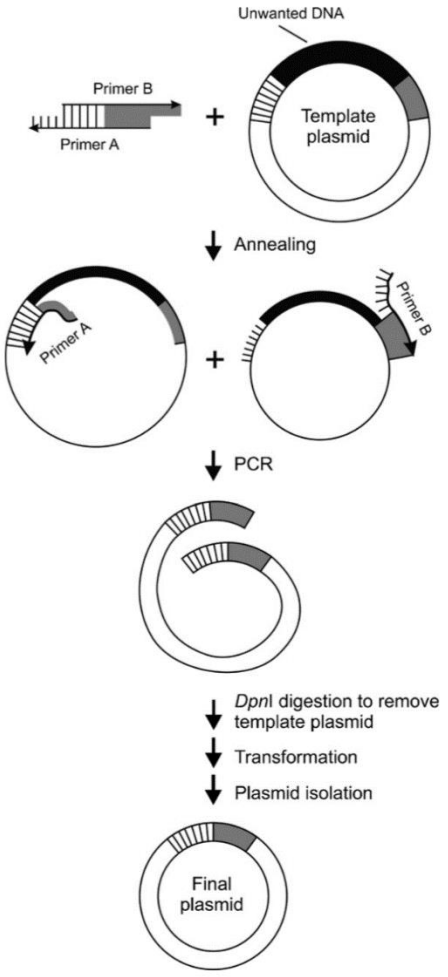


FIG 2 (6). Mechanism of PCR-mediated deletion using homologous-end designed primers. The dark region indicates area of intended deletion. Primers A and B have complementary regions with each other (indicated by gray and striped region). Primers bind template sequences via their 3' region while the 5' region dangles during PCR, which amplifies plasmid away from the region of intended deletion. PCR results in linear DNA product with homologous ends, which can join and circularize fragment by homologous recombination inside bacteria. Subsequent steps leading to the desired plasmid construct are indicated.

For transformation of linear PCR products into Subcloning Efficiency™ DH5α™ competent cells (Invitrogen), transformation and preparation of competent cells were performed as described by manufacturer's user manual.

Expression of YidC-GFP in BL21(DE3). Plasmid pEH1-YidC-GFP (4) was transformed into BL21(DE3). After successful transformation, overnight culture of the transformed BL21(DE3) was prepared using LB with kanamycin (30 µg/ml). The culture was grown overnight at with shaking (200 rpm) at 37°C. The next

day, 600 µl of the overnight culture was used to inoculate 30 ml of LB-kanamycin (30 µg/ml). Upon reaching an OD_{660} reading of 0.2, the culture was induced by IPTG at final concentration of 1 mM. Induced cultures were grown for 3 hours at 37°C with vigorous shaking (200 rpm).

Microscopy. Wet mounts of the bacterial culture were prepared without fixing the cells. 10-20 µl 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 Canon Powershot SD790 IS digital camera at final magnification of 1000x. Images were cropped and processed using ImageJ.

Polymerase Chain Reaction (PCR)-mediated gene deletion. The target region of deletion relative to the entire pEH-1 YidC GFP construct is shown in Figure 1a. Forward and reverse primers were designed to bind and amplify away from the P1 region of YidC, and to generate a linear product that has homologous ends (Figure 2), which can be circularized in DH5α by homologous recombination. Forward deletion and reverse deletion primers were designed to flank the coding region of P1 domain, upstream of nucleotide 78 (corresponding to amino acid residue 26) and downstream of nucleotide 960 (corresponding to amino acid 320) of the YidC-GFP construct. Specifications of primer design are described in Table 1. The annealing temperatures gradient between 40 to 70°C was used. The thermocycler of deletion PCR consisted of denaturing plasmid DNA at 95°C for 2 minutes, denaturing at 95°C for 30 seconds, annealing at 40 to 70°C for 30 seconds, extending at 72°C for 17 minutes. The PCR amplification was set to 30 cycles. To remove the original template plasmids, PCR product was immediately digested with DpnI for 1h at 37°C followed by an enzyme heat inactivation step for 20 minutes at 80°C. To evaluate the success of the PCR, products were run on a 0.8% agarose gel, stained with SYBR© Safe DNA stain at 180 volts for one hour in TAE buffer. Following confirmation, PCR products were purified using the PureLink PCR Purification Kit (Invitrogen). The purified pEH1-YidC ΔP1-GFP DNA were placed in aliquots and stored at -20 °C.

Table 1. Primers used in this study.

Primer	Sequence (5' – 3')	T_m (°C)	%GC	Length (nucleotides)
Deletion Fwd	CTGGGA**A**C AGGATGC**A** CCGCACCTG GATCTGC	68	68	32
Deletion Rev	CAGGTGCG G**T**GCATCCT G**T**TCCCAG GCTTGCC	67	60	32
Exterior Fwd	AGTCATCG CTTTGCTG TTCG	62	50	20
Exterior Rev	AGAGATGA ACCACAAC CAACC	61	48	21
Interior Fwd	GCCAGGGG AAACTGAT CTC	61	58	19
Interior Rev	CAGGGTGC TGTTCATC GC	62	61	18

Bolded nucleotides are introduced silent mutations (does not change translated amino acid) to compensate for self-dimer and secondary structure formation. Underlined nucleotide sequences of the forward are complementary to non-underlined sequences of the reverse, and vice versa. Non-underlined sequence binds to sequences on pEH-1 plasmid upstream or downstream of targeted deletion region.

Circularization of linear PCR products inside DH5α. DH5α can circularize linear DNA fragments that have homologous ends through homologous recombination (7). Linear pEH1-YidC-GFP with P1 deletion (PCR product) was used to transform the Subcloning Efficiency™ DH5α™ Competent Cells. 50 µl of the Subcloning Efficiency™ DH5α™ Competent Cells were mixed gently with 10 ng of the purified linear PCR product and incubated on ice for 30 minutes. The cells were then subjected to heat shock at 42°C for exactly 20 seconds. The tubes were immediately put on ice for two minutes. 950 µl of pre-heated LB broth was added to the cultures. The cells were allowed to recover at 37 °C for 1 hours at 225 rpm and subsequently plated on LB-kanamycin (30 µg/ml) plates to be grown overnight at 37°C. Isolated colonies were used to prepare an overnight culture of the DH5α bacterial cells in 5 ml LB with 50 µg/ml of kanamycin. The culture was grown overnight at 37°C in a shaker at 200 rpm. The next day, the plasmids were harvested using the Purelink Quick Plasmid Miniprep Kit (Invitrogen) and stored in aliquots at -20 °C.

PCR Deletion confirmation. PCR was used to confirm deletion of the targeted P1 region in YidC-GFP region of the pEH-1 plasmid. Two sets of primers, exterior and Interior, were designed to confirm the deletion of P1 domain. Exact sequences are shown in Table 1. The Interior forward and Interior reverse primers anneal to the inside of the P1 deletion, while the exterior forward and exterior reverse primers anneal to outside of P1 deletion. Platinum Taq (Invitrogen) was used. PCR was conducted by denaturing plasmid DNA at 94°C for 2 minutes, denaturing at 94°C for 30 seconds, annealing at 62°C for 30 seconds, and extending at 72°C for 70 seconds. The PCR amplification was set to 35 cycles. The amplified product was immediately run on a 1% agarose gel, stained with SYBR© Safe DNA Stain, at 180 volts for 30 minutes in TAE buffer.

Sanger Sequencing of deletion confirmation PCR products. PCR products of the deletion confirmation PCR, which used primers that anneal outside of P1 deletion and P1 deleted pEH-1 plasmid as templates, were sanger-sequenced at NAPS at UBC. The obtained DNA sequences were aligned to the DNA sequence of YidC T1 and P1 domain (reference), which was obtained from the NCBI database. Geneious® and its mapping algorithm was used to map and align sequences to the reference.

RESULTS

Wild-type YidC localizes at the poles of *E. coli*. Urbanus *et al*. used a YidC-GFP fusion protein to study distribution of YidC and observed preferential localization of YidC to the cell poles (4). Using the same construct, we planned to construct an in frame deletion of the P1 domain and ask whether or not this region of YidC is required for polar localization.

We began by establishing an experimental system to observed YidC-GFP expression in *E. coli* strain BL21(DE3). Figure S1 in supplementary information shows bright field and fluorescent images of the confirmation experiment. As the negative control, a sample of transformed BL21(DE3) that not induced by IPTG was imaged. No fluorescent signal was observed. Incubation of transformed BL21(DE3) in 1 mM IPTG resulted in fluorescent cells under the microscope. Both the cytosolic compartment and the cell membrane appeared fluorescent. For the vast majority of the fluorescing cells, fluorescent signatures concentrated at the polar ends of bacteria. In comparison, cytoplasmic fluorescence was significantly weaker. Our observation is

consistent with the results of

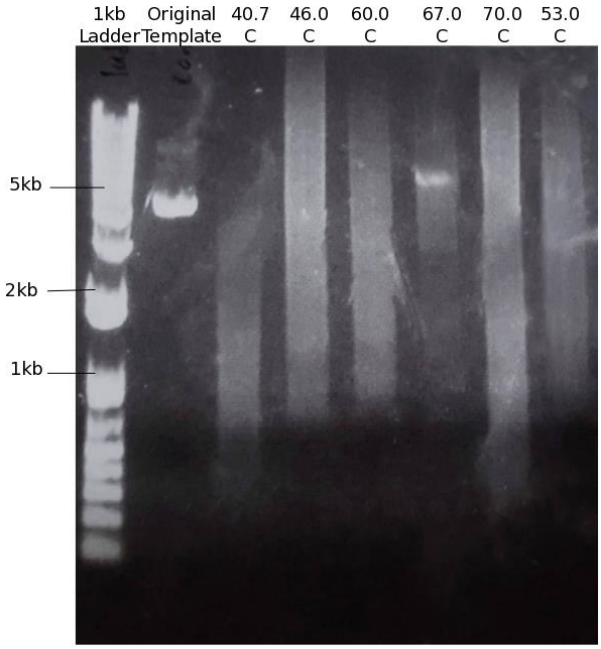


FIG 3. DNA gel of gradient PCR with homologous-end primer design. PCR was performed using homologous-end deletion primers, Pfu polymerase, and annealing gradient of 40 to 70°C. The original pEH-1 plasmid (template) and PCR products were analyzed by gel electrophoresis in 0.8% agarose gel (TAE). 180V (6V/cm) for 1 hour in 1x TAE buffer.

Urbanus *et al.* suggesting that YidC-GFP localizes to the cell poles (4).

Deletion PCR using homologous-end primers results in nonspecific products and the desired linear product containing P1 deletion. To delete the nucleotide sequence coding for amino acids 25-323 of YidC P1 domain (897 bp long), we used long-range PCR with homologous primers flanking the target region. To determine whether the PCR resulted in the desired linear product (5777 bp), the unmodified pEH-1 construct (6674 bp) was resolved on a gel. The desired PCR product was expected to migrate slower than the supercoiled pEH-1 construct. Figure 3 shows the unmodified pEH-1 plasmid migrated similarly to a 4kb linear DNA. All annealing temperatures except for 67°C resulted in a smear of PCR products. The annealing temperature of 67°C resulted in a linear DNA fragment of roughly 5500 bp, similar to the expected size of 5777 bp. However, subsequent series of PCR using annealing temperatures 60ºC to 70ºC failed to produce the specific PCR fragment (see supplementary figure S2). These results suggest that the desired linear pEH-1 construct was amplified using an annealing temperature of 67ºC.

***E. coli* strain DH5α is able to circularize linear PCR product by homologous recombination.** To circularize the linear PCR fragment containing the P1 deleted YidC-GFP, we purified and transformed the PCR product into competent *E. coli* strain DH5α. An attempt was made to transform using PCR product with and without DpnI digestion. Transformed DH5α were plated on kanamycin-Luria broth-agar plates. Only DH5α bacteria that circularize the linear pEH-1, which confers kanamycin resistance, should be able to grow. As the positive control, an aliquot of DH5α was transformed with the original pEH-1 plasmid was used. The DpnI-digested transformation resulted in a single colony while non-DpnI digested transformation resulted in 44 colonies (data not shown). Transformation efficiencies with DpnI digested and undigested PCR product were 9.5 $x10^4$ cfu/µg and 5.7 x 10^3 cfu/µg, respectively. These results suggest that the PCR product had been circularized in *E. coli* strain DH5α.

Circularized PCR fragment contains P1 deleted YidC-GFP. To determine if the circularized, modified pEH-1 plasmids contained the desired P1 domain deletion (25-323 aa), we first analyzed their size in comparison to the unmodified pEH-1, then performed PCR using primers that flanked the desired deletion by annealing outside the deleted region, and finally sequenced the PCR products. Transformation of DH5α using DpnI and non-DpnI digested PCR products resulted in a total of 45 isolated colonies. They were thought to contain the modified construct (YidC-GFP with P1 deleted). 15 colonies were sampled for analysis: 1 from the DpnI-digested transformation, and 14 from the non-DpnI-digested transformation. To analyze modified plasmids size, we harvested modified plasmid using PureLink plasmid miniprep kit and performed gel electrophoresis on 0.8% agarose gel for 120 minutes. As a control, we ran the original YidC-GFP containing pEH-1 plasmid in the same gel. The modified plasmid is expected to be around 1000 bp shorter than the control and thus should migrate faster and appear below the original plasmid in the gel. Of the 14 non-DpnI transformation colonies sampled, 3 did not show differences in migration pattern when compared to control (see supplementary Figure S3). 11 of the remaining constructs from non-DpnI digested transformation (construct #2, 3, 4, 5, 7, 9, 12, 13, 14, 15, 16) and the single construct from DpnI digested transformation (construct #1) were analyzed again alongside control group (the original plasmid). Figure 4 shows that all modified constructs contain plasmids roughly 1000 bp smaller than the original plasmid. Positive controls

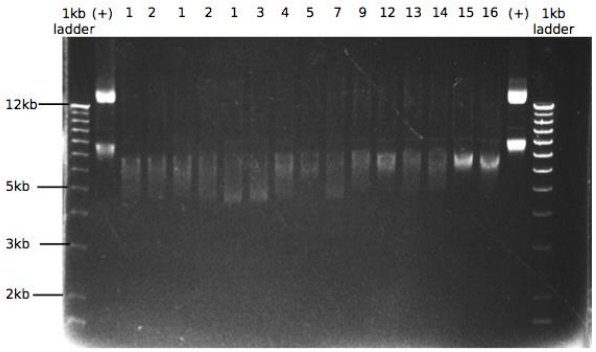


FIG 4. Size analysis of modified constructs thought to contain the desired deletion. pEH-1 plasmids that were circularized and maintained from the linear PCR product were harvested from DH5α and analyzed for size based on migration speed using gel electrophoresis. As a control, the original unmodified pEH-1 was also loaded. DNA samples ran in 0.8% agarose gel (TAE). 180V (6V/cm) for 120min in 1x TAE buffer.

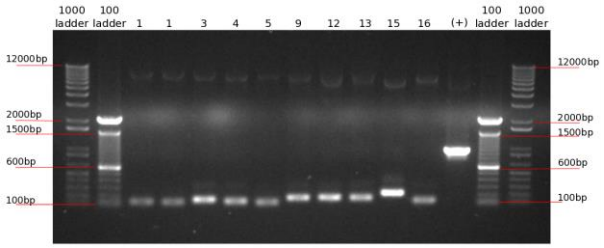


FIG 5. DNA gel of Hot-start PCR performed using exterior primers that flank the desired P1 deletion region. PCR was performed with Platinum Taq. PCR with the original unmodified plasmid construct as template was performed as a positive control. PCR products were analyzed by gel electrophoresis in 1% agarose gel (TAE). 180V (6V/cm) for 30 min in 1x TAE buffer.

appeared to contain another large DNA fragment in addition to the expected unmodified plasmid. Results indicate these twelve modified constructs could contain the desired P1 deletion (897 bp long).

To determine further that these twelve samples do have the desired deletion, we performed PCR using these constructs and the unmodified plasmid (positive control) as templates, and two sets of specially designed primers: one set flanking the P1 deletion (exterior primers), the other inside the P1 deletion (interior primers). PCR with exterior primers is expected to produce DNA fragments smaller than 100 bp when the modified constructs are used as templates. PCR with exterior primers and the original plasmids, without the deletion, is expected to produce fragments of around 1100 bp. As templates. PCR with forward exterior and reverse interior primers is expected to yield no products when a construct containing the correct P1 deletion is used as the template, and a product around 1000 bp when the original plasmid is used as the template. PCR was performed using Platinum Taq (Invitrogen) without provided enhancer solution. Products were analyzed by gel electrophoresis using 1% agarose gel. PCRs using forward exterior and reverse interior primer resulted in products around 1000 bp when the modified constructs and the original plasmid were used as templates, no difference was observed (see supplementary figure S4). Figure 5 shows that PCR using exterior primers only and the unmodified plasmid yielded DNA fragment around 1000 bp. All PCRs using exterior primers only and modified constructs resulted in fragments around 100 bp. Construct #15 produced a fragment that appeared to be larger than other samples. These results indicate the sample modified plasmids may contain the desired P1 YidC deletion coding for amino acids 23-325 of YidC.

To determine with certainty that the desired deletions are present in these sample plasmids, we selected four modified constructs (#1, 3, 12, 15) and the original plasmid as templates, and repeated the PCR described above using the exterior primer set. We submitted the PCR products for Sanger sequencing and analyzed the sequence results using Geneious®. Sanger sequencing results are described in Table S1 of supplementary information. Sequences of the PCR products, the deletion primers, and the exterior PCR primers were mapped against nucleotide sequence of YidC T1 and P1 domain from *E. coli* K-12 as the reference. To check if deletions were in-frame, the nucleotides were translated, and the amino acid sequences were analyzed. Supplementary figure S6 shows that PCR product from the original plasmid was mapped to the majority of the reference sequence as expected. The majority of P1 domain were observed to have been deleted in each of the four constructs. The four constructs (1, 3, 12, and 15) mapped towards the 3' region of reference very well, whereas the 5' region did not. There are variations between the four constructs. For example, the 5' region between the four constructs appeared variable. As shown in figure S6, 3' region of all four constructs aligned with high affinity to the reference. The exceptions were construct 3 and 15. Construct 15 had an additional 87 nucleotide fragment inserted at position 979. Construct 3 had an additional 22 nucleotide fragment that aligned by itself around position 910. Figure 6 shows the essentially same alignment as figure S6, but only construct 1 was aligned to the reference and compared to the intended deletion construct designed by us.

Deletion of YidC P1 domain affects localization of YidC. To determine if the deletion of P1 domain results in changes in localization of YidC-GFP, we harvested sample plasmids #1, 3, 12, and 15 from DH5α, transformed them

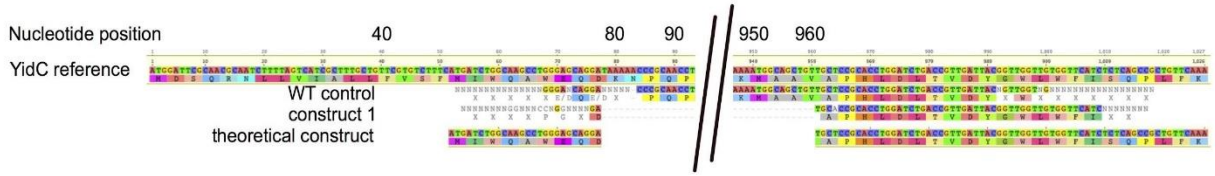


FIG 6. Nucleotide and protein sequence alignment of the ideal P1 deleted construct and construct 1 to YidC reference. DNA sequence of T1 and P1 domain of *E. coli* K-12 YidC (Gene ID: 948214) was used as a reference. PCR was performed using original pEH-1 (control) and modified constructs #1, 3, 12, 15 as templates, and primers flanking the P1 region. Exterior forward (nucleotide 24-43 of reference) and reverse (nucleotide 1079-1099 of reference) flank the P1 region (sequences not shown). PCR products Sanger sequenced from 5' to 3' using the exterior forward primer. Nucleotide sequences were aligned using Geneious, and then sequences were translated using +1 reading frame of the reference. Nucleotide and protein sequences that match with the reference are highlighted in color. Colorless regions of nucleotide and protein sequence did not match with the reference.

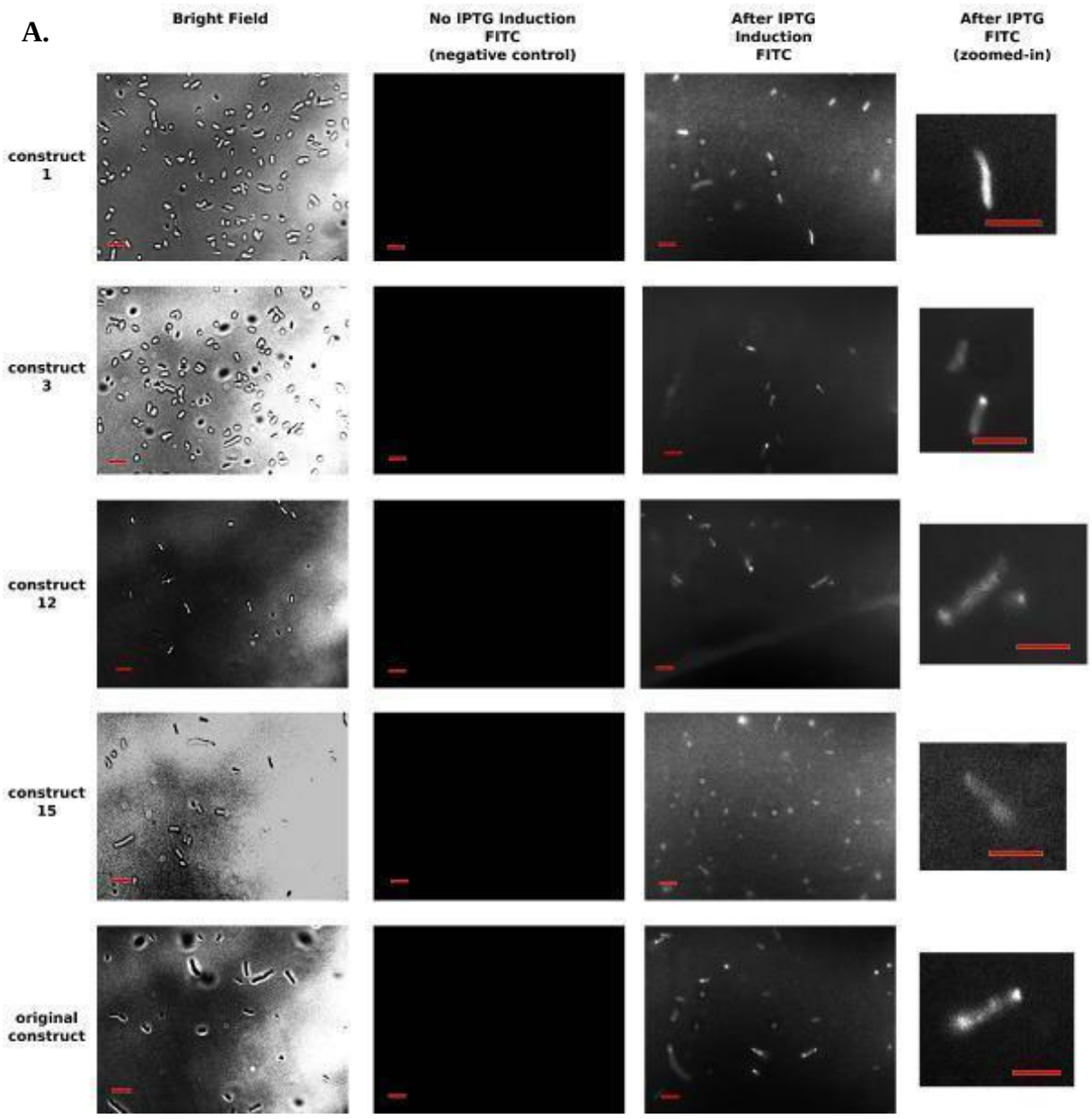
into BL21(DE3), prepared overnight culture in kanamycin Lysogeny broth, induced expression of P1-deleted YidC-GFP protein by 1 mM IPTG induction for 3 hours, and performed fluorescent microscopy on wet mount samples. As the negative control, a replicate of BL21(DE3) transformants was not induced by IPTG. As the positive control, the original pEH1 plasmid containing undeleted YidC-GFP protein was transformed into an aliquot of BL21(DE3), induced by 1 mM IPTG for 3 hours, and fluorescent images taken at 1000x magnification. Figure 7A shows that all negative controls (IPTG uninduced) did not display any fluorescent signatures, as expected. BL21(DE3) with original construct resulted in bacteria expressing fluorescent signatures throughout the cell; most of them had signatures localized at their poles. Constructs 1, 3, 12, and 15 resulted in bacteria expressing GFP also, but only some of them had signatures localize at their poles. Figure 7B shows the percentage of fluorescent bacteria that had signatures localize at the poles for each experimental groups (percentage ± 95% confidence interval, n = 123 to 165). The original construct resulted in higher percentage of bacteria with polarized fluorescent signatures. The deletion of P1 domain resulted in a lower percentage of bacteria with polarized fluorescence. These data suggest that the P1 domain could play a role in polar localization of YidC.

DISCUSSION

YidC is an essential inner membrane protein responsible for the folding and insertion of proteins into the membrane (1). YidC P1 is the largest of the periplasmic domains of YidC and is located between transmembrane domains 1 and 2. Its function is not fully understood. The removal of this domain does not impact YidC protein function (1), but it is likely to be involved in interaction with other proteins because it is largely hydrophilic and extends out from the rest of the protein, according to the crystal structure of YidC (2). A previous study suggested YidC preferentially localizes at the polar ends of bacteria (4), but its mechanism of action is not known.

In this study, we hypothesize that the P1 domain is involved in localizing YidC to the cell poles. We created an in-frame deletion of most of the P1 domain in the YidC-GFP fusion protein to study the role of P1. The resulting plasmids were transformed in *E.* coli strain BL21(DE3) to determine if the YidC P1 domain is necessary for the localization of the YidC protein to the poles. To answer our research questions, we had to first make a novel YidC-GFP construct with the desired mutation, sequence the constructs to verify that the deleted region was present and in-frame, and finally evaluate how the deletion affected localization. We initiated this study by repeating an experiment done by previous researchers showing that in a *E.* coli strain expressing a YidC-GFP fusion protein, the fluorescent signal localizes to the cell poles. Shown in Figure 3, fluorescence was detected at the poles of the cells induced with IPTG. In the negative control, where the cells were not induced with IPTG, no fluorescence was detected. These data show that the YidC-GFP fusion protein is expressed and localizes to the cell poles in a similar manner to what was observed by Urbanus *et al* (4). An interesting observation is that some GFP accumulate around the membrane of the cell. One explanation could be YidC protein inserts into the bilayer at the poles and then diffuses away from the poles.

Deletion construct was successfully created but after many difficulties. The most important step in the study is achieving the deletion of P1 domain in YidC. The key feature of the primer deletion design (Table 1) is the complementarity between the primers, which results in homologous ends in the final PCR product. This complementary, alongside high GC content and long primer length, results in very high melting temperature and makes the primers bind very easily to nonspecific regions. For the deletion PCR, an annealing temperature gradient between 40 to 70°C was used. Shown by Figure 3, analysis of PCR products indicates that a faint band appeared in only lane 6. Reaction was conducted with an annealing temperature of 67 °C. The approximate size of this PCR product is 5500 bp, which is very similar to the desired linear product of 5777 bp. The intended deletion is roughly 900bp long, and the pEH-1 plasmid deduced shown by figure 1a is roughly 6700bp. These results show that PCR-based deletion strategy using homologous primer pair may be able to produce the desired deletion product, but mostly such PCR reactions produce nonspecific products, which are likely due to the high GC content and a high annealing temperature of the YidC P1 domain. This has an important implication in that the single band observed in lane 6 may not be homogenous. There may be variability in the sequences because the primers bind easily to non-specific regions. Although we were unable to reproduce this band in subsequent PCRs, the product from the first PCR should be sufficient for subsequent experiments. The PCR products contain the modified YidC-GFP protein within pEH-1 plasmid sequence that has become linearized as a result of PCR. DH5α is observed to be able to circularize linear DNA fragments that have compatible homologous ends in a *rec*A independent mechanism (7). However, the recombination event is not very specific and can result in sequence variability and even the order at which sequences are joined (6). For this study, the linear PCR products was transformed into competent DH5α cells and plated. Some linear PCR product were then digested with DpnI prior to transformation. DpnI digestion removes only template DNA as the enzyme only cuts methylated strands (PCR products are not methylated). All PCR except the first PCR had DpnI digestion done immediately after the cycles. We extracted products without DpnI digestion for the first deletion PCR, and we obtained the desired PCR product. Some were left undigested because the DpnI



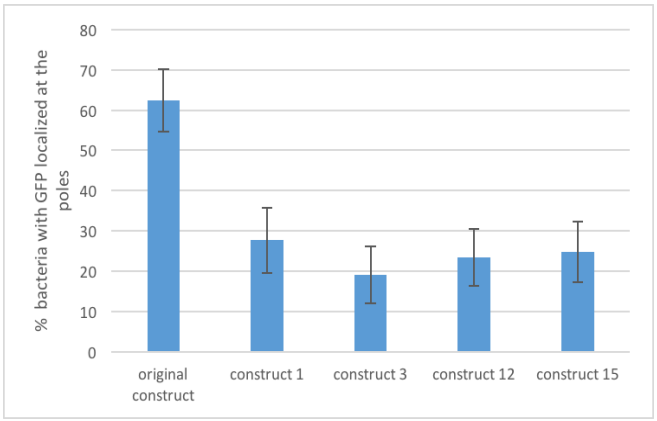


FIG 7. Localization of Four different P1-deleted YidC-GFP in BL21(DE3). (a) Four modified pEH-1 constructs (#1, 3, 12, 15), each contains a different P1-deleted YidC-GFP fusion protein under lac-T7 promoter, were transformed into BL21(DE3). GFP fusion protein was expressed by incubating an overnight culture of transformed BL21(DE3) in 1 mM IPTG for 3 hours. Bright field and fluorescent images (FITC) were taken at 1000x magnification. Samples were not fixed; mounted by placing the sample on a microscope slide and covered by glass cover slip. Scale bars = 2 um. (b) Fluorescent images of each experimental group were analyzed. The total number bacteria that expressed fluorescent signatures and the number of bacteria that also had signatures localized at the poles were counted in ImageJ. Results were expressed as the percentage of those that had fluorescent signatures at the poles compared to the total number of all fluorescent bacteria. (Percentage ± 95% confidence interval, n = 123 to 165)

enzyme buffer contained bovine serum and was thought to be able to interfere with transformation. This explains why some plasmids extract from DH5α seemed to contain the original plasmid (Supplementary figure S3); those cells were likely to have received the PCR template (the original pEH-1) instead of the PCR product. The transformation efficiencies were very low in comparison to positive control done with the circular original pEH-1 plasmid. This may be due to the fact that DH5α must obtain successfully circularized linear plasmid in addition to maintaining the plasmid and expressing kanamycin resistance gene. The Figure 4 gel results indicate the linear PCR product were circularized and smaller compared to the original unmodified pEH-1 plasmid. This suggests that these modified constructs may contain the desired deletion.

To further confirm that the constructs contain the desired deletion, we used PCR analysis. Two set of deletion-confirmation primers were designed and ordered. Interior primers amplify the region within the targeted P1 deletion. Exterior primers have complementarity to the region slightly outside of the region to be deleted and amplifies towards the P1 region. The circularized plasmid was harvested and amplified with the exterior primer pairs. As seen in Figure 5, only a small band of around 100 bp is seen in all the experimental lanes. The 100 bp bands seen are regions external to the deletion, which was amplified by the primers. In the control lane, a band larger than 900 bp is present. The 900 bp band shown in the positive control lane shows the presence of the YidC P1 region, which was expected. No 900 bp bands were seen in any of our experimental lanes, indicating that the deletion of P1 region was successful.

From all the possible modified pEH-1 constructs (annotated as constructs #1, 2, 3, and so on), four were chosen for DNA sequencing. As shown by Figure S6, all four constructs (1, 3, 12, 15) had the P1 deletion mutation. From the alignments and translation of aligned nucleotide sequence, we conclude that the deletions were likely in-frame: protein sequence shortly before and after the intended P1 deletion region agree with that of the YidC reference, for each of the constructs. Construct 1 bore the largest deletion. Constructs 3, 12 and 15 contained deletions which varied in the number of nonspecific nucleotide fragments introduced by the editing process in addition to the P1 in-frame deletion leading to ambiguous nucleotide sequences and length. Construct 15 contains a large nucleotide region (87bp) inserted at around nucleotide number 979. This 87 nucleotide region aligned with 57% identity towards the head of the reference YidC P1 domain (see supplementary figure S5). Construct 3 had a unique 22 bp fragment around position 910. However, upon examination of the sequence, the first 14 nucleotide of this fragment are identical to a region of construct 3 that aligned to positions 961 to 974 (Figure S6). The variations in nucleotide sequence and additional fragment insertion are likely due to the deletion strategy used. The deletion primers have complementary regions with binding interactions towards upstream and downstream regions of the area to be deleted resulting in two potential areas of binding instead of one. Primer sequences are also high in GC content and long in length. These features of the primer result in nonspecific PCR products, and when this is combined with the variable nature of homologous recombination in *E. coli*, variations are created; this possibly allowed for some nonspecific PCR products to circularize in DH5α. In addition, as shown in Figure S6, two point mutations were observed at positions 72 and 964, where G was mutated to A and T to A, respectively. They are silent mutations intentionally introduced during primer design to compensate for self-dimer formation of the deletion primers. For all the Sanger sequencing results, the 5' region often failed (Figure S7). From the sequencing results, we were able to conclude that we had created a construct (plasmid #1) where the P1 domain was deleted in-frame and with almost no undesired nucleotide fragments.

Expression modified YidC-GFP suggests P1 domain may play a role in localizing YidC to the poles of *E. coli.* Our data from Figure 7a provides preliminary evidence that the YidC P1 domain is required for localization of the YidC protein to the poles of *E. coli*. As seen by fluorescence microscopy of cells with P1 deletion constructs fused to GFP, there was no specific polar localization of GFP within most cells. Instead, GFP was detected throughout the entire cell and in the cytoplasm. Concentrating of fluorescence at the polar ends, which was observed when YidC-GFP with P1 was expressed, was observed at a significantly lower frequency after P1 was deleted from YidC-GFP. This indicates the P1 domain may be involved in localizing YidC to the polar ends and distributing YidC within bacteria. Looking at the original construct (positive control) we are able to see the obvious localization of GFP at the poles of the cells. Figure 7b suggests that the observed difference in where GFP localizes may be statistically significant. IPTG induced cells with deletion constructs yielded a lower percentage of cells with GFP localization at the poles. Localization at the poles of cells of P1 deletion is around half of the original construct. In all deletion constructs, we are able to detect a substantial decrease in GFP polar localization, as seen in Figure 7b. These results provide support that YidC P1 region may be required for polar localization of YidC.

In conclusion, our findings identify the importance of YidC P1 periplasmic domain in the localization of the YidC protein. Following in frame deletion of the P1 region from pEH1 YidC-GFP plasmid we observed a change in the localization of GFP under microscopy. Our

study highlights the importance of the P1 domain in the proper localization of YidC protein, and suggests that it may play an essential role in the localization of YidC protein to the poles of *E. coli*.

FUTURE DIRECTIONS

One can characterize the P1-deleted YidC-GFP construct made in this study. This can be accomplished by obtaining a YidC knockout *E. coli* strain, and then attempt to complement it with our P1-deleted YidC-GFP constructs. In this study, we did not attempt to delete background YidC that BL21(DE3) naturally expresses from its own genome. Another improvement would be in microscopy techniques. Fixing of bacteria onto microscope slide may improve image quality in microscopy by inhibiting bacteria from moving, and allow for the analysis of the percentage of total bacteria present that actually expressed GFP. We did not attempt to fix samples before microscopy.

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