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Development of Tools for Genomic-Editing of the P1 Periplasmic Region of YidC: CRISPR/Cas9 Coupled with Lambda Red Recombineering in *Escherichia coli* MG1655.

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SUMMARY YidC is a conserved and essential protein that plays a role in the insertion and folding of Sec-dependent and Sec-independent membrane proteins. It has six transmembrane domains and one large 35 kDa periplasmic domain (P1) positioned between transmembrane domains 1 and 2. Although YidC is found across all domains of life, only P1 seems to be evolutionarily conserved among Gram-negative bacteria, suggesting that the YidC P1 domain serves an important function. Previous studies have attempted to study the P1 domain of YidC by using P1-specific antisense RNA to knock out expression of YidC; however, this did not eliminate leaky expression of YidC nor allow specific study of the P1 domain. To create a clear genetic background for the study of the P1 domain of YidC, this study aimed to couple Lambda Red Recombineering (LRR) with CRISPR/Cas9 to create a scarless genomic deletion of the P1 domain of YidC. Sequential transformation of LRR and CRISPR/Cas9 machinery into *Escherichia coli* MG1655 resulted in numerous viable colonies. However, further investigation via colony PCR and Sanger sequencing showed inconclusive results as to whether or not a successful P1 deletion in YidC was generated. Furthermore, while Sanger sequencing indicated successful assembly of CRISPR guide RNA, the CRISPR/Cas9 activity of our cloned pCRISPR appears to be ineffective at killing wild type cells, suggesting that CRISPR/Cas9 effectiveness is highly dependent on the target site of the gRNA. This study provides useful insight for futures studies hoping to make genomic deletions in essential genes using the CRISPR/Cas9 system in tandem with Lambda Red Recombineering.

INTRODUCTION

Integral membrane proteins are essential for cellular life, as such, organisms have evolved mechanisms to assemble and insert these proteins into the membrane. YidC is a conserved and essential protein found in all domains of life that is involved in the folding (chaperone activity) and insertion (insertase activity) of Sec-dependent and Sec-independent membrane proteins (1). YidC is 548 amino acids long with six transmembrane domains spanning the inner plasma membrane and one periplasmic domain (P1). The P1 domain is approximately 35 kDa and positioned between transmembrane domains 1 and 2, as illustrated in Figure 1 (2). Interestingly, although YidC homologs exist across all domains of life, the P1 domain is only conserved in Gram-negative bacteria, suggesting that the YidC P1 domain may play a unique role in these bacteria. In *Escherichia coli*, sec-dependent membrane proteins have been shown to require YidC for proper folding and functioning (3,4), such as: MalF and LacY, used for the transport of maltose and lactose, respectively, and penicillin binding proteins (PBPs), which are involved in peptidoglycan synthesis. While it is clear that YidC homologs carry out an important and essential role in survival of organisms, the roles of the individual YidC domains, such as the P1 domain conserved in Gram-negative bacteria, and mechanistic details of YidC function are not well characterized.

The essential nature of YidC makes it difficult to study using classical techniques, and while some groups have managed to study YidC using conditional knockouts and mutations studies, the results have yet to clearly define a role for YidC, or the P1 domain. Conditional knockdowns of YidC using antisense RNA (asRNA) suggested that YidC does not seem to play a role in maintaining cell shape, since cells containing the asRNA construct do not form a distinct filamentous phenotype, but instead maintained a phenotype consistent to that of the

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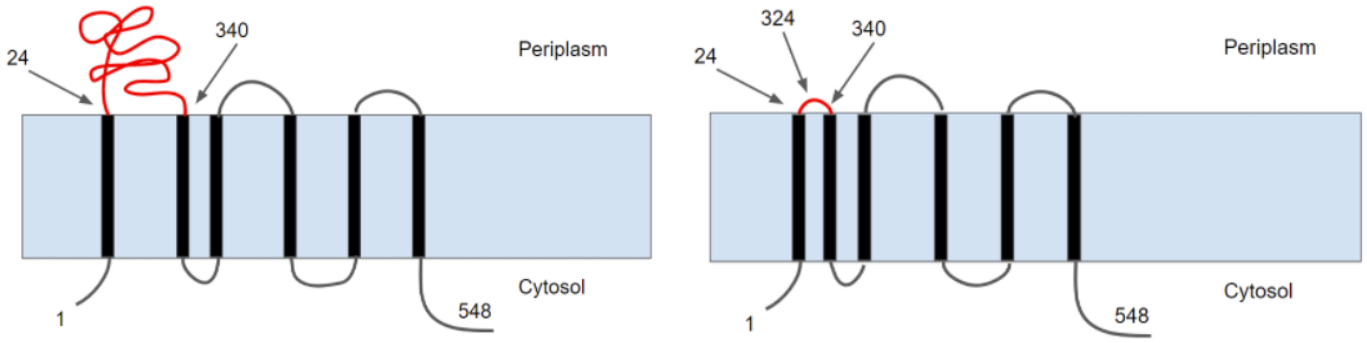

FIG. 1 Diagram of YidC transmembrane and periplasmic domains. (A). Six transmembrane domains are indicated in black with P1 periplasmic domain shown in red. (**B**). YidC protein after deleting the dispensable regions of P1.

wild type (5). However, YidC appeared to have an effect on cell growth, as cells containing the asRNA construct showed a reduced growth rate at 30˚C, compared to at 37˚C (5). Furthermore, deletion studies have shown that 90% of the P1 domain is dispensable for the viability of *E. coli* (resides 25 to 323) (6). These studies have created a foundation on which the function of YidC, and particularly the P1 domain, can be further studied. Herein, we attempted to use a combination of Lambda Red Recombineering (LRR) with CRISPR/Cas9 selection to generate a clean deletion of the P1 domain of YidC in *E. coli.* Studying the function of the YidC P1 domain will give us a better understanding on the significance on why only P1 appears to be conserved across Gram-negative bacteria.

METHODS AND MATERIALS

Bacterial strains and growth conditions. *E. coli* strains and the plasmids used in this study are listed in Table 1. *E. coli* MG1655 was kindly provided by the Department of Microbiology & Immunology, University of British Columbia. *E. coli* MG1655 strains were grown in Luria Broth (LB) medium for 16 to 18 hours at 37˚C (for strains not containing pKD46) or 30˚C (for strains containing pKD46), while shaking at 200 rpm. LB recipe was obtained from Sambrook and Green (7). LB agar medium contained 1.5% agar. Where required, media were supplemented with the appropriate antibiotics at the following final concentrations: 100 µg/ml ampicillin (amp) for pKD46, 50 µg/ml kanamycin (kan) for pCRISPR, and/or 25 µg/ml chloramphenicol (cam) for pCas9.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description[1]	Source or reference
E. coli strains		
MG1655	F- λ- *ilvG rfb50 rph-1*	11
Plasmids		
pEH-1-YidC-GFP-Urbanus	*yidC::gfp*	12
pKD46	*gam exo beta* P_{araB} amp^R Temperature sensitive (only grows at 30˚C)	David Oliver
pCas9	*cas9* cam^R	Addgene; Cat# 42876
pCRISPR	gRNA; kan^R duplicated BsaI sites	Addgene; Cat# 42875

[1]Amp^R, ampicillin resistance; Cam^R, chloramphenicol resistance; Kan^R, kanamycin resistance

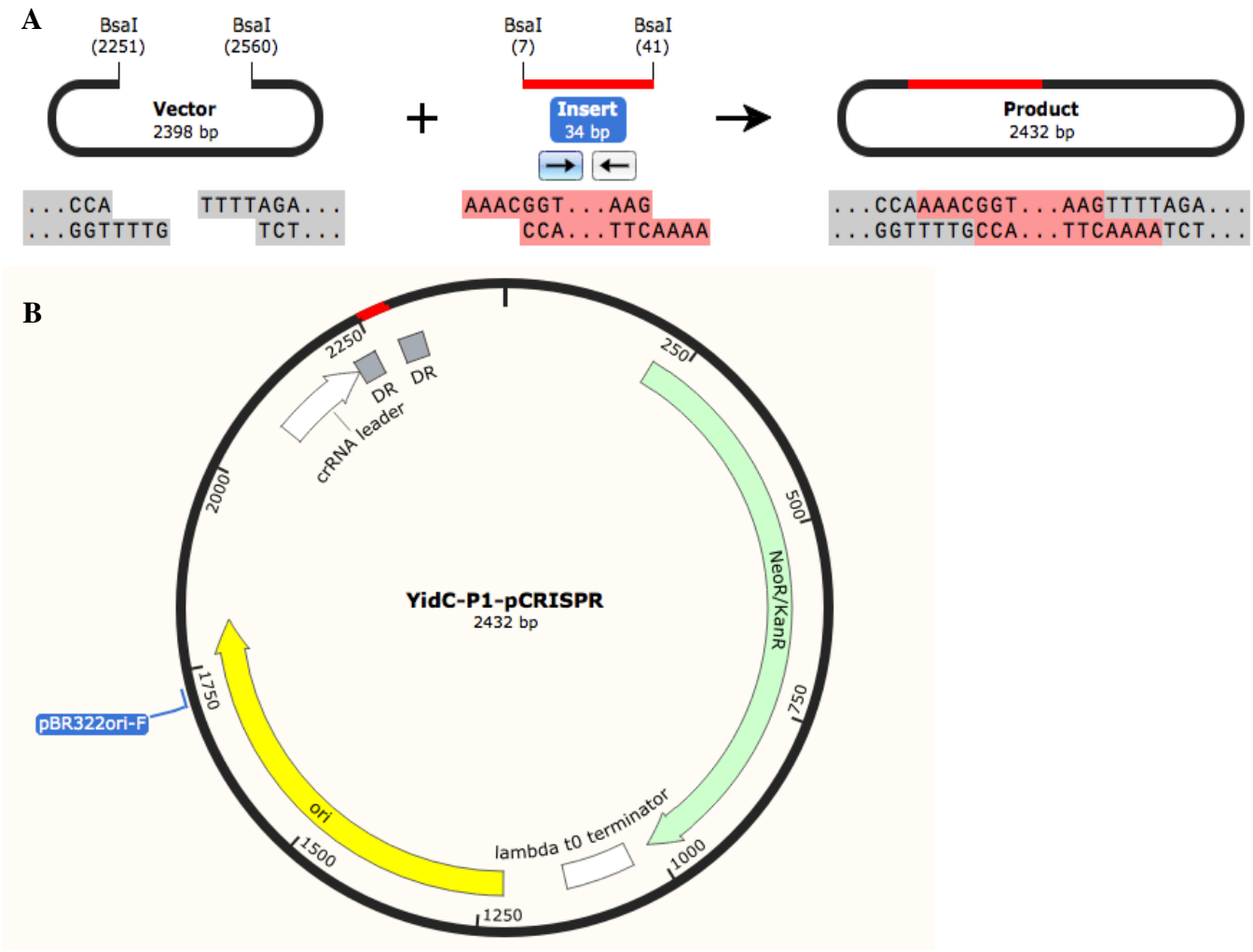

FIG. 2 Construction of YidC-P1-specific pCRISPR. (**A**) BsaI restriction enzyme digested pCRISPR and YidC-P1-specific gRNA oligos designed with overhangs for BsaI ligation. Nucleotide sequence of YidC-P1 (Gene ID: 948214) was obtained from NCBI database, and pCRISPR plasmid (Addgene #42875) was imported from Snapgene (Marraffini Lab). (**B**) YidC-P1-specific pCRISPR construct with the cloned gRNA situated in between the direct repeats shown in red. Universal primer pBR322ori-F was used to sequence across pCRISPR to confirm the presence of gRNA insert. pCRISPR plasmid was imported from Snapgene (Marraffini Lab).

Isolation of pEH-1-YidC-GFP-Urbanus, pKD46, pCas9, and pCRISPR. *E. coli* containing pKD46 and pEH-1-YidC-GFP-Urbanus were obtained as colonies on LB agar. pCas9 and pCRISPR were obtained as *E. coli* stab cultures (Addgene). Plasmid DNA was isolated from overnight cultures using the Purelink Quick Plasmid Miniprep Kit (ThermoFisher) according to the manufacturer's instructions. Purified plasmids were quantified using a Nanodrop 2000 (Thermo Scientific) and stored at -20˚C.

Preparation of $CaCl_2$ competent *E. coli* MG1655 cells. *E. coli* MG1655 overnight cultures were inoculated at 1% into 100 mL of fresh LB and incubated at 37˚C at 200 rpm. After 3 hours, the cultures were chilled on ice for 20 minutes, harvested by centrifugation at 4000 rpm for 10 minutes, and resuspended in 20 mL of 0.1M $CaCl_2$. Cells were then incubated on ice for 30 minutes, pelleted by centrifugation at 4000 rpm for 10 minutes, and resuspended in 5 mL of cold 0.1M $CaCl_2$ with 15% (v/v) glycerol. Resulting competent cells were stored in 50 µL aliquots at -80˚C.

Transformation of $CaCl_2$ competent *E. coli* cells MG1655. For transformation of chemically competent *E. coli*, 100 ng of DNA was incubated on ice with 50 µL of competent cells for 30 minutes. Cells were then heat shocked in a 42˚C water bath for 30 seconds,

followed by incubation on ice for another 2 minutes. Cells were then added to 200 µL of LB broth and shaken for an hour at 200 rpm at 30˚C, before being plated on LB agar plates containing appropriate antibiotics. pKD46 and pCas9 were heat-shock transformed together into chemically competent MG1655 cells. The duo-transformed cells were then made chemically competent again, followed by heat-shock transformation of cloned pCRISPR and the YidC-ΔP1 dsDNA template into these cells. Transformed pKD46 and pCas9 cells were plated on LB agar supplemented with ampicillin and chloramphenicol. Triple-transformed cells were plated on LB agar supplemented with ampicillin, chloramphenicol, and kanamycin. Plates were incubated at 30˚C for 20 hours before being transferred to 4˚C.

Construction of YidC-P1-specific pCRISPR. YidC-P1 guide RNA (gRNA) insert was cloned into pCRISPR according to the pCRISPR spacer cloning protocol provided by Addgene (8). Oligonucleotide sequences used to construct gRNAs are listed in Table 2. gRNA inserts were prepared by phosphorylating single stranded oligonucleotides using T4 PNK (New England Biolabs), followed by annealing by incubating oligonucleotides for 5 minutes at 95˚C and allowing it to cool to room temperature overnight. pCRISPR was digested by BsaI (New England Biolabs), dephosphorylated by CIAP (ThermoFisher), and then purified with the Purelink PCR Purification Kit (ThermoFisher). Annealed gRNA inserts were ligated into prepared pCRISPR using T4 ligase (New England Biolabs). Resulting mixture was transformed into chemically competent *E. coli* DH5α and plated onto LB agar. Plasmid DNA from five colonies for each pCRISPR construct were isolated. pCRISPR constructs were sequenced with universal primer pBR322ori-F to confirm insertion of the gRNA template. nBLAST was used to map and align the sequencing results to the DNA sequence of the YidC P1 domain (Gene ID: 948214) obtained from the NCBI database.

Fusion PCR for generation of YidC-ΔP1 dsDNA template. Fusion PCR was used to create homologous recombination templates. PCR fragments were amplified from pEH1-YidC-GFP-Uranus template DNA with the primer pairs listed in Table 3. The primer sequences are

Table 2. Primers and oligonucleotides used in this study

Primer/Oligonucleotide	Sequence (5'-3')	T_m (˚C)	%GC	Length (nucleotides)
gRNA 1a	AAACGGTCGTGATGGCCCGGATAACCCGGCTAAG	69.1	58.8	34
gRNA 1b	AAAACTTAGCCGGGTTATCCGGGCCATCACGACC	68.0	55.9	34
gRNA 2a	AAACACTTCTATACCGCTAATCTGGGTAAG	57.8	40.0	30
gRNA 2b	TTACCCAGATTAGCGGTATAGAAGTCAAAA	57.4	36.7	30
YidC-ΔP1 FE	CGGACCATTTGATACCAGAGAACACTAACGATGG ATTCGCAACGCAATCTTTTAGTCATC	67.8	43.3	60
YidC-ΔP1 RE	CCGCCCTTATTTTAGCGAAAACTCACCGAATCAGG ATTTTTTCTTCTCGCGGCTATGCAG	69.4	46.7	60
YidC-ΔP1 R	TGGATCCATTTCAGCAGTTTGAACAGCGGCTGAG AGATGAACCACAACCAACCGTAATCA	70.5	46.7	60
YidC-ΔP1 UP	GGA GGA GGA GGA GGA CCA GGC TTG CCA GAT CAT GAA AGA CAC GAA CAG CAA AGC GAT	79.5	54.4	57
YidC-ΔP1 DN	TCC TCC TCC TCC TCC GCA GCT GTT GCT CCG CAC CTG GAT CTG ACC GTT GAT TAC GGT	74.2	59.6	57
pBR322ori-F	GGGAAACGCCTGGTATCTTT	55.1	50.0	20

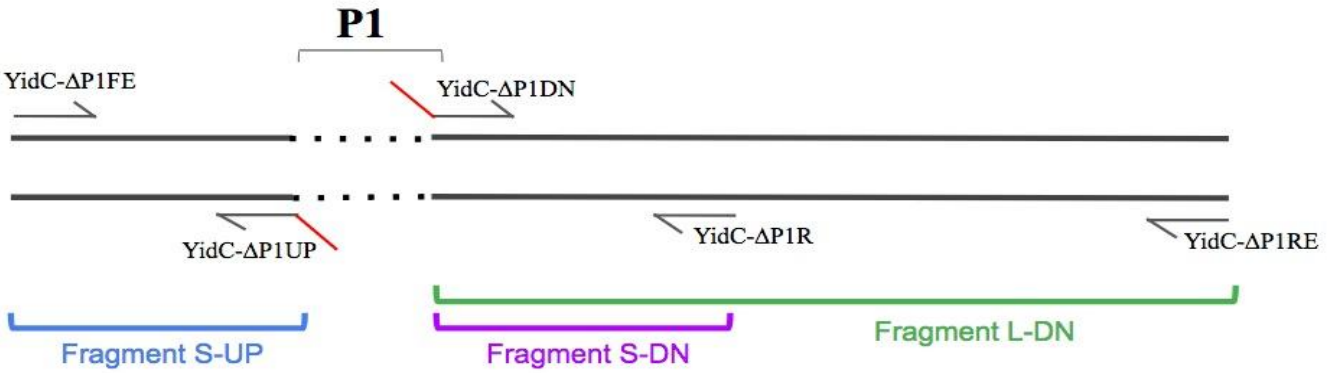

FIG. 3 Outline of primers used in fusion PCR to create the YidC-ΔP1 dsDNA template. Primers used to generate fragments S-UP, S-DN, and L-DN by gradient PCR. Red region indicates 3' complementary sticky ends engineered into primers YidC-ΔP1 UP and YidC-ΔP1 DN used for the annealing of UP fragments and DN fragments.

shown in Table 2. PCR was performed using Pfx polymerase (ThermoFisher) according to the manufacturer's instructions. 30 PCR cycles were run with denaturation performed at 94˚C for 15 seconds followed by the three annealing temperatures 70˚C, 66.1˚C, or 60.7˚C for 30 seconds, and extension at 68˚C for 90 seconds. Resulting PCR fragments were used to assemble homology directed repair (HDR) templates via fusion PCR. Parameters kept the same as gradient PCR except an annealing temperature of 60˚C was used. Resulting templates were visualized by DNA gel electrophoresis, purified, quantified, and stored at -20˚C. Correct assembly of the HDR templates was confirmed by sequencing the purified PCR products.

Colony PCR of triple transformants to confirm genomic P1 deletion of YidC. Colony PCR was performed to screen for colonies containing the recombinant YidC-ΔP1. The primers used were YidC-ΔP1FE and YidC-ΔP1RE, as listed in Table 2, which anneal to the ends of the *yidC* gene. Colony PCR was carried out using the same mixture and cycle parameters as in fusion PCR, with the exception that a colony dab was used as template DNA instead of adding purified template DNA to the reaction mixture. As a negative control to ensure transformed cells contained all three plasmids, *E. coli* cells, each containing different antibiotic markers, were plated on LB plates supplemented with ampicillin, chloramphenicol and kanamycin. Cells plated include: the triple-transformed cells (containing pKD46, pCas9, and pCRISPR), the duo-transformed cells (containing pKD46 and pCas9), pKD46-containing *E. coli* cells, pCas9-containing *E. coli* cells, pCRISPR-containing *E. coli* cells, and sensitive *E. coli* cells. Plates were incubated at 30˚C for 20 hours before being transferred to 4˚C.

Gel purification, PCR amplification, and Sanger Sequencing to confirm identity of colony PCR products. Following DNA gel electrophoresis of colony PCR products, agarose gel extraction and purification of the two different colony PCR products was carried out using the PureLink Quick Gel Extraction Kit (ThermoFisher). The purified products were then used as template DNA for PCR amplification using primers that flank the ends of the *yidC* gene, YidC-ΔP1FE and YidC-ΔP1RE, as listed in Table 2, in order to generate enough product for sequencing. The resulting amplified PCR products were purified using Invitrogen PureLink® PCR Purification Kit (ThermoFisher), and the concentration and purity of the product was determined using the Nanodrop spectrophotometer (Thermo Scientific). Both purified PCR products were sanger-sequenced at Genewiz at UBC with primers YidC-ΔP1FE and YidC-ΔP1RE, as listed in Table 2, that anneal at the ends of the *yidC* gene. BLAST was used to map and align the sequencing results to the DNA sequence of the *yidC* gene (Gene ID: 948214) obtained from the NCBI database.

Confirming CRISPR/Cas9 activity. Cloned pCRISPR was heat-shock transformed without the YidC-ΔP1 dsDNA template into chemically competent MG1655 cells containing pKD46 and pCas9. Transformed cells were plated on LB agar supplemented with ampicillin, chloramphenicol, and kanamycin. Plates were incubated at 30˚C for 20 hours before being transferred to 4˚C.

Table 3 Primer pairs used to amplify the individual fragments to use in fusion PCR.

Fragment Name	Primers	Expected size (bp)
L-DN	YidC-ΔP1 DN + YidC-ΔP1 RE	741
S-DN	YidC-ΔP1 DN + YidC-ΔP1 R	107
S-UP	YidC-ΔP1 FE + YidC-ΔP1 UP	114
L-ΔP1	YidC-ΔP1 FE + YidC-ΔP1 RE	840
S-ΔP1	YidC-ΔP1 FE + YidC-ΔP1R	206

RESULTS

Fusion PCR product contains an in-frame YidC-ΔP1 deletion. To construct a dsDNA template for LRR mediated HDR, we made an in-frame YidC-ΔP1 deletion using fusion PCR. Regions of *yidC* upstream and downstream of P1 were PCR amplified with complementary overhangs to create a P1 deletion. After resolving the PCR products via gel electrophoresis, the gel showed bands that resolved at approximately 110 base pairs for S-UP and S-DN, as expected, as shown in Figure 4A, and bands that resolved at approximately 740 base pairs for L-DN, as expected, as shown in Figure 4B. Using the amplified fragments, fusion PCR was carried out to anneal and amplify the L and S fragments via the complementary overhangs. As shown in Figure 4C, after resolving the fusion PCR products via gel electrophoresis, the gel showed bands that resolved at approximately 840 base pairs for L-ΔP1, as expected, and bands that resolved at approximately 206 base pairs for S-ΔP1, as expected, thus indicating successful generation of HDR templates. Subsequent sequencing of L-ΔP1 and S-ΔP1 confirmed that the correct HDR templates were generated, as shown in the supplemental material. Structure of YidC containing the P1 deletion is shown in Figure 1.

Some pCRISPR constructs contained the YidC-P1-specific gRNA. gRNA insert was successfully cloned into digested pCRISPR. This step is essential for CRISPR/Cas9 to be used effectively as a selection tool, since a gRNA is required for Cas9 to make a site-specific double bond lethal cleavage at the genomic deletion site. gRNA inserts were designed to recognize the YidC P1 deleted site in the genome in order to guide Cas9 to render cells containing wild type genomic *yidC* non-viable. In order for Cas9 to cut, it also requires the presence of a specific three-nucleotide 5'-NGG-3' protospacer adjacent motif (PAM) located downstream of the target sequences (9).

Triple-transformants contain pKD46, pCas9, and pCRISPR. Following the heat-shock transformation of cloned pCRISPR and YidC-ΔP1 dsDNA template into duo-transformed cells containing pKD46 and pCas9, plates yielded colonies too numerous to count, as shown in Figure 5A. This was a very unexpected result. As previous studies have shown low recombination efficiency by LRR, we had originally expected a small number of cells to have been successfully recombined and selected for by CRISPR/Cas9 activity which renders wild type cells nonviable. The extensive number of colonies made us speculate that pCRISPR may not have been transformed into MG1655 to guide Cas9 endonuclease activity. To set up a control to test for the success of transformations, the triple-transformants, as well as double and single transformants were streaked onto LB agar supplemented with chloramphenicol, ampicillin, and kanamycin. The triple-transformants contain all three antibiotic resistance markers and were expected to grow. Double and single transformants did not contain all three antibiotic resistance plasmids and were expected to show no growth. Figure 5B shows growth in the triple transformants only and no growth for the double and single transformants, therefore confirming the presence of all three plasmids in the triple-transformants.

CRISPR/Cas9 activity does not render wild type cells nonviable. To confirm that the cloned pCRISPR was able to make a lethal double stranded breakage at the wild type YidC-

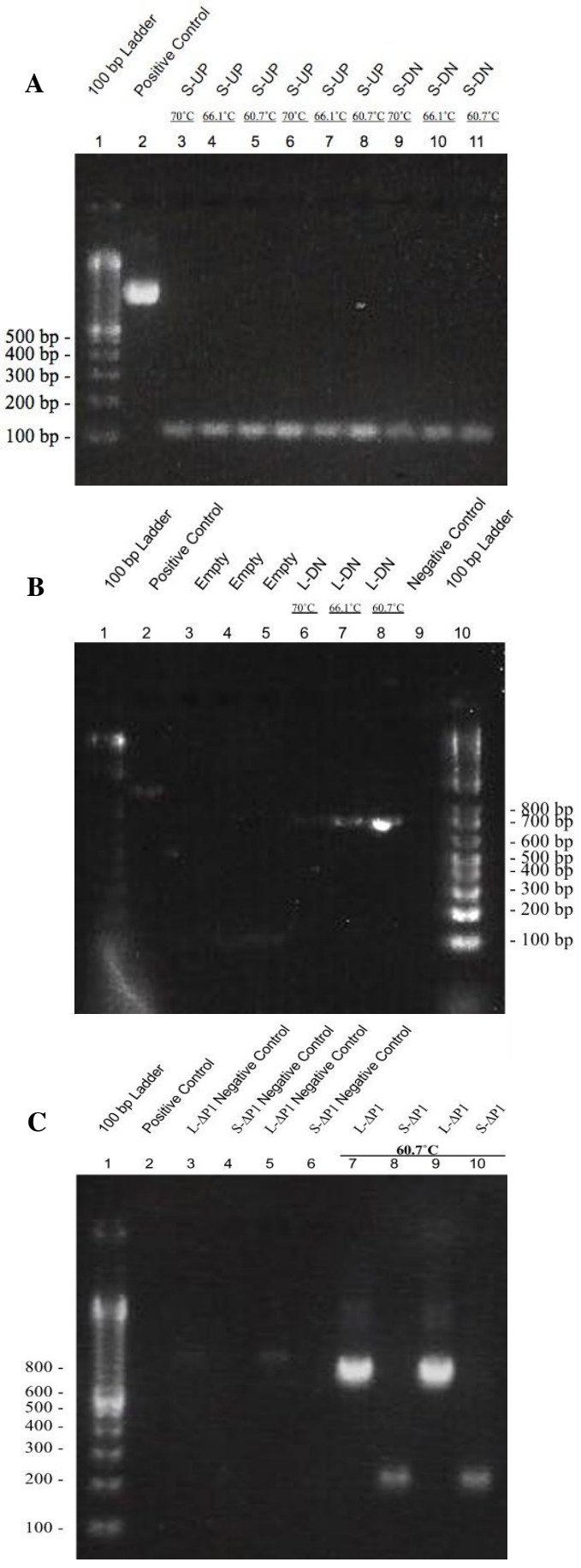

FIG. 4 A) **Agarose gel electrophoresis of gradient PCR amplified short fragment products upstream and downstream of P1.** Gradient PCR was performed with Platinum Pfx using annealing temperatures of 60.7˚C, 66.1˚C, and 70˚C. PCR products were analyzed by gel electrophoresis in 2% agarose gel (Fast Blast DNA Stain) ran at 60V for 1 hour in 1X TBE buffer. *WZAK30* DNA template was used as the positive control. **B**) **Agarose gel electrophoresis of gradient PCR amplified long fragment products downstream of P1 domain.** Gradient PCR was performed using platinum Pfx using annealing temperatures of 60.7˚C, 66.1˚C, and 70˚C. PCR samples were analyzed in 2% agarose gel (Fast Blast DNA Stain) ran at 60 V for 1 hour in 1X TBE buffer. *WZAK30* DNA template was used as the positive control. **C**) **Agarose gel electrophoresis of fusion PCR amplified YidC-ΔP1 dsDNA constructs.** Both the long and short YidC-ΔP1 dsDNA constructs was generated using fusion PCR. Fusion PCR was performed using platinum Pfx at an annealing temperature of 60.7˚C. 0.5 uL DNA template was used in lanes 7 and 8, while 1.5 uL DNA template was used in lanes 9 and 10. Lane 3 to lane 6 are the negative controls with no Pfx polymerase added. Fusion PCR products were analyzed by gel electrophoresis in 2% agarose gel (Fast Blast DNA Stain) ran at 60 V for 1 hour in 1X TBE buffer.

P1 domain, we heat-shock transformed our pCRISPR constructs into duo-transformed chemically competent cells containing pKD46 and pCas9 without the YidC-ΔP1 dsDNA template and plated these cells onto LB agar supplemented with ampicillin, chloramphenicol, and kanamycin. Since no template DNA was present, no LRR should have occurred, and the transformed cells were expected to show minimal or no growth if CRISPR/Cas9 activity was effective in rendering wild type cells nonviable. Our results yielded colonies too numerous to count, thereby suggesting that the CRISPR/Cas9 activity of our pCRISPR constructs were ineffective, since no lethal double-stranded breakage was made in cells containing the wild type YidC-P1 domain, and wild type cells remained viable.

Colony PCR of triple-transformants with YidC-ΔP1 dsDNA template showed inconclusive results on the presence of genomic P1 deletion. Colony PCR was conducted to verify the presence of a genomic P1 deletion in triple-transformed *E. coli* MG1655 cells containing the YidC-ΔP1 dsDNA template. Triple-transformed colonies were re-streaked three times for pure isolated colonies prior to performing colony PCR. Gel electrophoresis of the colony PCR products revealed two distinct DNA bands for each of the sixteen tested colonies, corresponding to ~800 base pairs and ~1647 base pairs. The upper band ("WT" band) corresponded to size of the *E. coli* MG1655 positive control, while the lower band ("insert" band) corresponded to the size of the YidC-ΔP1 dsDNA insert, as shown in Figure 6. The identities of these bands were confirmed via gel excision, gel purification, and PCR amplification of both bands. Interestingly, the gel electrophoresis of these amplified PCR products both revealed a single bright band that corresponded to the size of the YidC-ΔP1 dsDNA insert, although the PCR product amplified from the "WT" band also showed a subtle upper band that may correspond to the *E. coli* MG1655 wildtype *yidC*, as shown in Figure 7. The lower band was also less prominent in the lane containing the "WT" band amplicon. This may indicate possible contamination during gel excision, which caused the preferential amplification of the shorter YidC-ΔP1 dsDNA insert. To further support this theory, we sent both PCR products to be sanger-sequenced and obtained sequencing results that were identical to the YidC-ΔP1 dsDNA insert for both products. Based on these results, it is inconclusive whether or not LRR succeeded in generating the genomic P1 deletion.

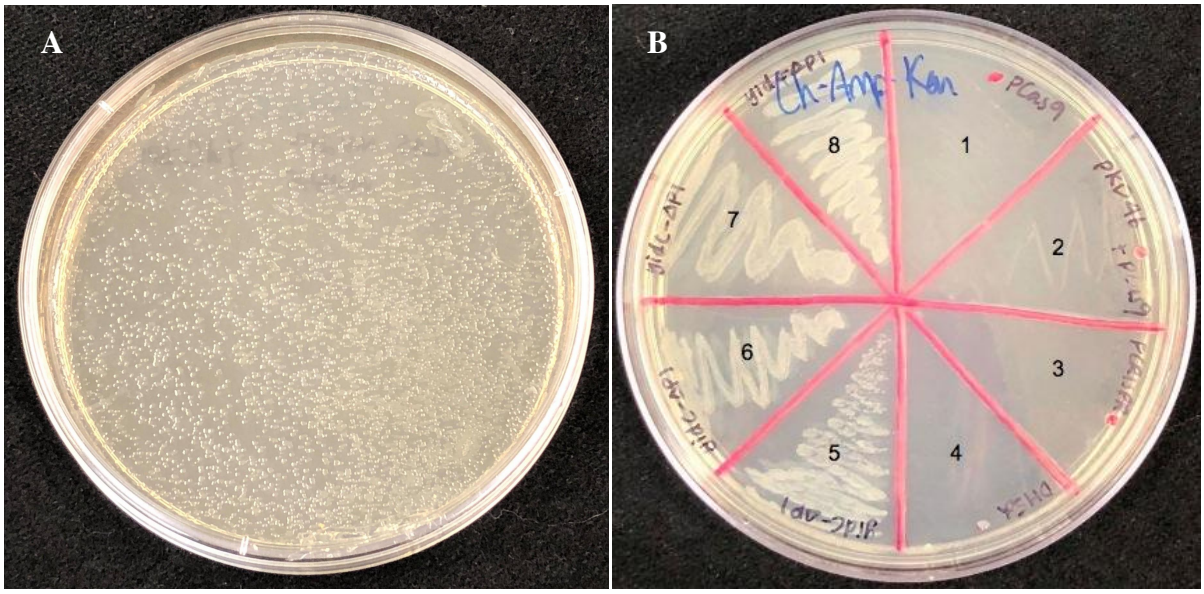

FIG. 5 Triple MG1655 *E. coli* transformants containing pKD46, pCas9, pCRISPR, and YidC-ΔP1 dsDNA template. Negative control for testing the presence of plasmids. A) Colonies too numerous to count were observed from the transformation of pCRISPR and YidC-ΔP1 dsDNA into duo-chemically competent MG1655 *E. coli* containing pKD46 and pCas9. **B**) *E. coli* cells containing plasmids with different antibiotic markers were plated on LB agar supplemented with antibiotics cam, amp, and kan. Section 1 was streaked with pCas9 containing cells with cam resistance. Section 2 was streaked with pCas9 and pKD46 double transformant cells with cam and amp resistance. Section 3 was streaked pCRISPR containing cells with kan resistance. Section 4 contains sensitive *E. coli* DH5α cells. Sections 5 to 8 contain triple-transformed cells with cam, amp, and kan resistance.

DISCUSSION

This study creates a foundation on which further study of the P1 domain of YidC can be studied. More specifically, we attempted to create a clean genomic deletion of the P1 domain of YidC by coupling LRR with CRISPR/Cas9 selection.

To successfully couple the LRR system and CRISPR/Cas9 system to generate a P1 deletion in YidC, we had to generate a couple of novel tools: (1) a YidC-ΔP1 dsDNA template for LRR, (2) a YidC-P1-specific pCRISPR to successfully select for recombinant colonies, and (3) pKD46 and pCas9 chemically competent duo-transformants. We successfully generated the YidC-ΔP1 dsDNA template via fusion PCR. Sanger sequencing confirmed an inframe P1 deletion in YidC, with the last 25 amino acids of the P1 domain left intentionally undeleted to allow active YidC function. We also successfully constructed YidC-P1-specific pCRISPR by cloning gRNA 1 into pCRISPR and sequencing across the gRNA insert to confirm cloning success. When YidC-P1-specific pCRISPR and YidC-ΔP1 dsDNA templates were transformed into the pKD46 and pCas9 duo-transformants, a large number of triple-transformants were observed. The oddly high transformation efficiency led to the speculation of whether the YidC-P1-specific pCRISPR effectively guided Cas9 endonuclease activity to select for YidC-ΔP1 recombinants. An interesting observation of the triple-transformants was that they were all uniformly small-sized, which supports previous studies' observations of significantly reduced colony size in CRISPR/Cas9 selected recombinants (10). To verify that recombinants contained a genomic P1 deletion in *yidC*, colony PCR was used to amplify the genomic *yidC* of triple-transformant colonies. The gel electrophoresis of these colony PCR products resulted in two bands that possibly corresponded to wildtype and mutant copies of *yidC*. Speculating that the observation of double bands was due to the formation of colonies comprised of both wild type cells containing *yidC* and recombinant cells containing *yidC-ΔP1*, triple-transformant colonies were restreaked to for pure isolated recombinant colonies prior to re-attempting colony PCR. We expected to observe only one band corresponding to the YidC-ΔP1 dsDNA template in the subsequent gel electrophoresis analysis of these colony PCR products; however, the gel electrophoresis of purified colony PCR products showed that double bands were retained across all of the screened colonies.

Potential explanations for the presence of two *yidC*-constructs per colony. Of the fifteen unpurified triple-transformants that had the genomic *yidC* amplified, the two bands were consistently observed in the DNA gel (data not shown). The presence of the two bands was initially thought to be due to the formation of mixed colonies made up of wild type cells containing *yidC* and recombinant cells containing *yidC-ΔP1*. This speculation arose from the fact that although the YidC-P1-specific pCRISPR construct was inefficient at eliminating wild type *yidC* cells, as shown by the presence of growth in the negative control of the triple-transformants lacking YidC-ΔP1 dsDNA template for LRR, the possibility of a single recombinant colony being generated was not eliminated entirely. However, the genomic amplification of *yidC* using restreaked colonies continued to show a retention of the two bands when analyzed by gel electrophoresis, as shown in Figure 6. Across the eight of the sixteen purified colonies in Figure 6, the upper band of colony 6 corresponding to the size of wild type MG1655 *yidC* was evidently less bright while the lower band corresponding to the size of the YidC-ΔP1 insert was the brightest compared across all the other colonies. This suggests that colony 6 may have been the desired recombinant colony containing genomic *yidC-ΔP1*.

Since the mixed colony theory was shown to be inconclusive, another possibility for the presence of two *yidC*-constructs in each colony may be due to an excess of YidC-ΔP1 dsDNA template present on the plates and/or on the cells. This may be due to a contamination of the pipettes with the template, therefore allowing the transfer of template to the plates and the PCR tubes. As a result, the shorter YidC-ΔP1 dsDNA template gets preferentially amplified due to its smaller size compared to the original longer wild type YidC template.

There may also be a biological explanation for the presence of two *yidC*-constructs in each colony. This theory suggests that perhaps *yidC* was duplicated in the genome rather than deleted; in other words, *yidC* may have been so essential for the survival of the cell that biological mechanisms may prevent *yidC* from being deleted from the genome, thus resulting in two copies of *yidC* rather than one. To investigate this, a Southern blot may be carried out on restreaked colonies to check for inconsistencies in genomic DNA sizes.

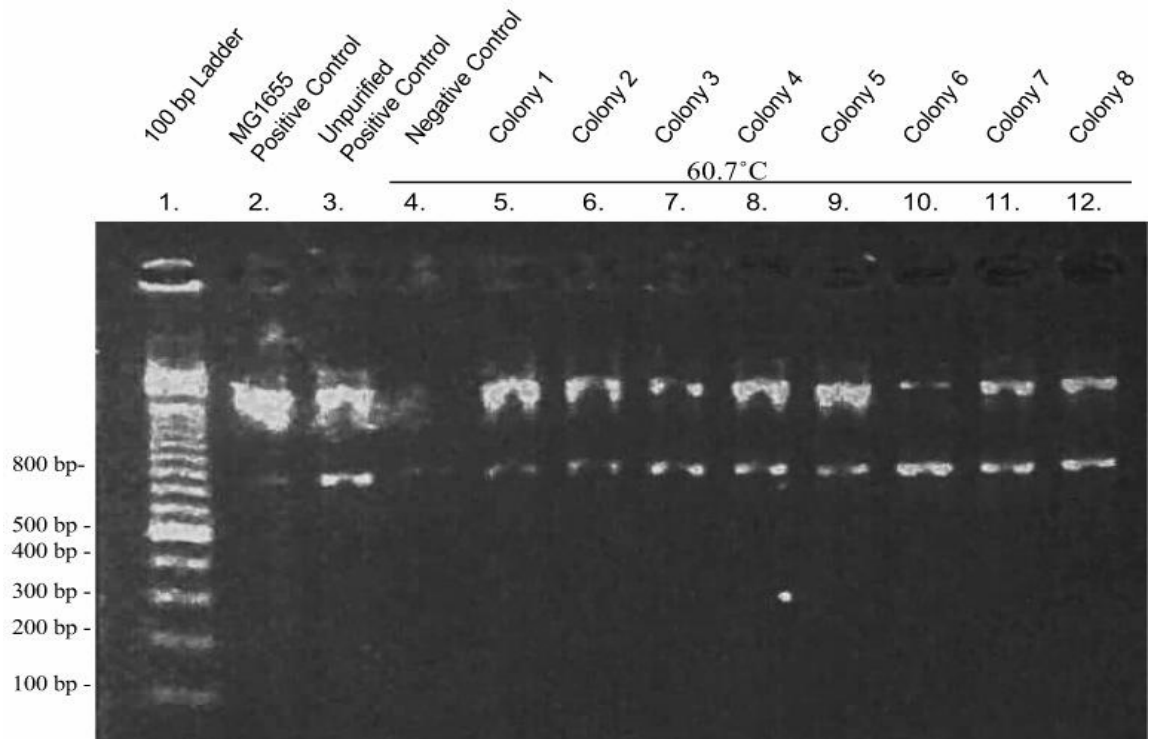

FIG. 6 Agarose gel electrophoresis of colony PCR products performed using restreaked MG1655 *E. coli* triple-transformant colonies. Colonies contain pKD46, pCas9, pCRISPR, and YidC-ΔP1 dsDNA constructs. Positive controls used were the MG1655 *E. coli* and the un-restreaked triple-transformant colony. Negative control was the absence of DNA template replaced with water. Across all sixteen colonies that were restreaked (eight shown), two visible bands were observed with the upper band corresponding to MG1655 and lower band corresponding to the YidC-ΔP1 insert. Colony PCR products were analyzed in 2% agarose gel (Fast Blast DNA Stain) ran at 60 V for 1 hour in 1X TBE buffer.

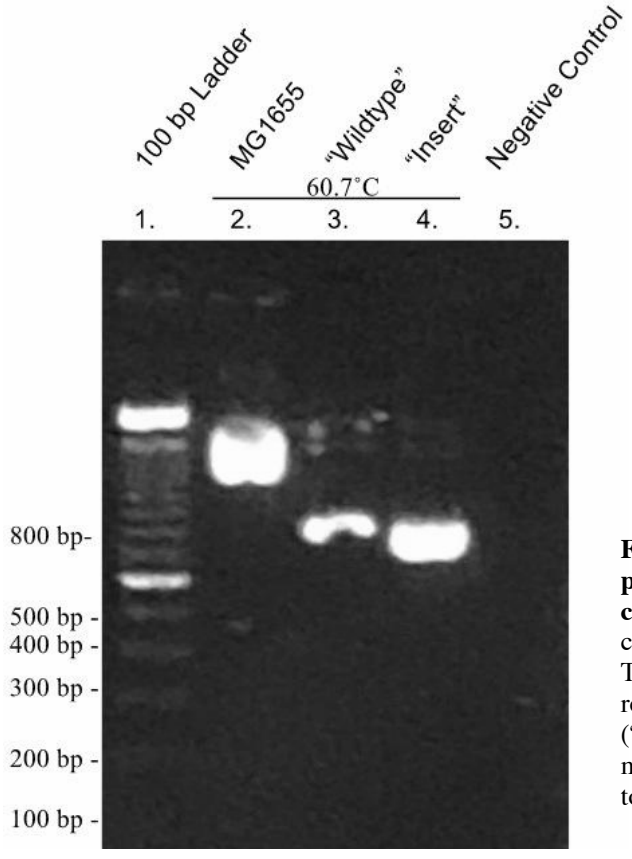

FIG. 7 Agarose gel electrophoresis of gel excised PCR products of restreaked MG1655 *E. coli* triple-transformants colonies. Presence of insert band was visible in the lane corresponding to the excised "wildtype" band and "insert" band. The intensity of the lower band ("insert") in lane 3 is significantly reduced by half compared to that of lane 4 while the upper band ("wildtype") in lane 3 is more visible than that of lane 4. This may suggest cross-contamination during gel excision process due to the preferential amplification of the shorter YidC-ΔP1 insert.

CRISPR gRNA may dictate Cas9 cutting efficiency. Our pCRISPR construct Y1 contained the YidC-P1-specific gRNA insert; however, activity of the CRISPR/Cas9 system was not observed. Using the CHOPCHOP scoring algorithm, it revealed that the Y1 gRNA we designed had an efficiency of approximately 0.52, which is relatively low (10). Previous data reveals that GC-rich gRNAs improved targeting efficiency, whereas U-stretches close to the PAM sequence resulted in gRNA with low efficiencies. This observation is a result of early termination in gRNA transcription due to the presence poly-U bases, which resembles the transcription termination sequence (11). The GC content is about 58% for gRNA 1a and about 55% for gRNA 1b, which is not high enough to give optimal gRNA efficiency. *E. coli* has been noted to survive active target sites by CRISPR/Cas9 at certain sequence positions (12). The lack of cleavage can also be attributed to the activation of the SOS response and repair via the homologous recombination pathway, therefore allowing *E. coli* to remain viable against weak self-targeting CRISPR/Cas9 systems (12).

Studies have stated that the transformation efficiency of *E. coli* using a plasmid carrying a CRISPR system is decreased by 1000X fold (12). It is also noted that the plasmid-mediated delivery of the CRISPR/Cas9 system components does not eliminate the possibility of random plasmid sequence integration into the host genome (13). Aside from the gRNA-dictated Cas9 cutting efficiency, the endonuclease activity may be improved by introducing a protein-mediated delivery system. Previous studies have also demonstrated that the delivery of Cas9:sgRNA protein complex significantly reduced off-target activity of Cas9 (14). The inefficient Cas9 activity observed in our results may necessitate the need to use a protein-mediated delivery of the CRISPR/Cas9 system components.

In conclusion, our study has produced two tools to be used for future research: a YidC-ΔP1 dsDNA template, and MG1655 cells transformed with pKD46 and pCas9. Our results also revealed the presence of wild type YidC and YidC-ΔP1 constructs in our final transformants, which we hypothesize is due to dsDNA template contamination of pipettes. A third tool, YidC-P1-specific pCRISPR, was produced but display little activity, possibly due to an inefficient gRNA design.

Limitations Due to time constraints of the course, we did not have the opportunity to create more efficient gRNA oligos using gRNA algorithmic scoring tools like CRISPRscan and CHOCHOP, (10). With more time, it would have been possible to create several new gRNA-pCRISPR constructs with higher efficiencies using the gRNA design tools. In addition, we would have liked to perform our experiment using isolated Cas9 proteins instead of expression through a pCas9 plasmid. With an efficient YidC-P1-specific pCRISPR, we could then carry on to perform effective selection of triple-transformed cells containing genomic *yidC-ΔP1*. If time permitted, we would have also induced pKD46 with arabinose as pKD46 contains an arabinose regulated promoter for the expression of Lambda Red genes (γ, β, exo). This would have increased recombination frequency of the YidC-ΔP1 template into genomic DNA.

Future Directions Using novel scoring algorithm gRNA design tools like CRISPRscan and CHOPCHOP, several new YidC-P1-specific gRNAs can be designed to enhance the guiding activity of pCRISPR in order to increase the chance of selecting for a mutant (10). Since a plasmid-mediated delivery system does eliminate the possibility of plasmids integrating into the host genome, protein-mediated delivery of Cas9 protein or Cas9-gRNA complex can be attempted as it has been shown to reduce the off-target effects of Cas9 as well (14). To obtain a more definitive answer to the presence of the two YidC-P1 constructs in triple-transformants, a primer that flanks outside of *yidC* in MG1655 genome should be used to determine if YidC-ΔP1 insert was indeed recombined into the host genome. Using the duo-transformed *E. coli* cells produced in this study, newly designed YidC-P1-specific pCRISPR constructs can be tested. For the duo-chemically competent cells containing pCas9 and pKD46, they can be arabinose induced in order to increase the expression of LRR homologous recombination proteins. To increase recombination efficiency, a shorter single stranded DNA template could be substituted for the double stranded DNA template. A suitable control to test for the effectiveness of the CRISPR/Cas9 and LRR selection tool would be to repeat the experiment on a non-essential gene like *lacZ* in *E. coli.* The design of

the mutant *lacZ*-specific dsDNA template can include an engineered green fluorescent protein to permit an easy screening to *lacZ* mutants. Using IPTG and X-Gal, this would also allow an easy visual determination of the efficiency of CRISPR/cas9 system by screening for presence of *lacZ* mutants.

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