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Ribosomal 16S rRNA Sequencing Shows That A Putative *yid*C *Escherichia coli* Knockout Strain Is Actually *Rhizobium*

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To study the function of the major periplasmic domain of the inner membrane insertase YidC, Choi *et al.* constructed two chromosomal *yid*C knockout strains of *Escherichia coli,* WJKC-1 and WJKC-2. WJKC-1 strain contains pEH1-*yidC-GFP* plasmid, which encodes the entire *yidC* gene. WJKC-2 strain contains the pYidC-*Δp1-GFP*-Xu plasmid, which lacks the major periplasmic domain of YidC. Preliminary growth experiments suggested a temperature sensitive phenotype as these strains grew at 20°C and 30°C but not at 37°C. Here we investigate the location of the chloramphenicol (*cam*R) cassette by polymerase chain reaction. The PCR experiments demonstrated proper primer binding to the positive controls, but only nonspecific binding to WJKC-1 and WJKC-2 genomic DNA. The sequencing results obtained from the junction between *camR* cassette and the neighboring gene, *mnmE*, lead us to investigate the possibility of *Rhizobium* species. 16 SSU rRNA amplification and sequencing confirmed that WJKC-1 and WJKC-2 are indeed *Rhizobium* species.

YidC is a 60kDa essential inner membrane protein in Gram-negative bacteria. It functions as a chaperone in cooperation with the Sec translocon and as an independent insertase to facilitate the folding and translocation of membrane proteins (2). YidC comprises of six transmembrane helices with a 35kDa periplasmic domain (P1) between transmembrane domains 1 and 2 (2). The function of YidC P1 domain is not well understood. To study the first periplasmic domain of YidC, two chromosomal *yid*C knockout strains of *Escherichia coli* WJKC-1 and WJKC-2 were constructed by Choi *et al.* (1).

To construct the chromosomal deletion, both strains were initially complemented with pEH1 plasmid encoding a kanamycin resistance gene and a *yid*C variant fused at its C-terminus to green fluorescence protein (GFP) (1). WJKC-1 strain contains the pEH1-*yidC-GFP* plasmid, which encodes the full-length *yid*C, and WJKC-2 contains the pYidC-*Δp1-GFP*-Xu plasmid which lacks the P1 domain (3). Choi *et al.* utilized lambda-red recombination system to introduce a 649 base pair chloramphenicol cassette flanked with regions of *yid*C (1). Following the selection of the *E. coli yidC* knockout strains WJKC-1 and WJKC-2, growth experiments were performed. Initial observations indicated growth at 20°C and 30°C but not at 37°C. Attempts at confirming the genomic deletion of *yid*C via amplification of *cam*R cassette insertion were not successful (1). Thus, we sought to follow up this study by genetically characterizing strains WJKC-1 and WJKC-2.

We hypothesized that the process of creating a *yid*C chromosomal knockout in WJKC-1 and WJKC-2 had resulted in the disruption of genes in close proximity to *yid*C via polar effects, thus causing a temperature sensitive phenotype. However, using a combination of polymerase chain reaction (PCR) and Sanger sequencing, we found that these strains are *Rhizobium* rather than *E. coli*.

MATERIALS AND METHODS

Bacterial strains, growth, and media. The WJKC-1 and WJKC-2 strains used in this investigation were *E. coli* BL21(DE3)-derived strains constructed by Choi *et al.* (1). Strains WJKC-1, WJKC-2, BL21 and plasmid pKD3 were obtained from the Microbiology & Immunology Department Strain Collection at the University of British Columbia. Strains were grown in Luria-Bertani (LB) broth or agar containing 25μg/mL chloramphenicol (CAM), 10μg/mL kanamycin (KAN), and 15mM isopropyl β-D-1-thiogalactopyranoside (IPTG). WJKC-1 and WJKC-2 were grown at 30°C. *E. coli* BL21 and *E. coli* DH5α containing pKD3 plasmid were grown at 37°C. LB medium was prepared in deionized water with the addition of 10 g/L NaCl, 10 g/L tryptone, and 5 g/L yeast extract. If solid medium was required, 12.5 g/L of agar was also added.

Growth of WJKC-1 and WJKC-2 at different temperatures. Strains were incubated at 20°C, 30°C, and 37°C for up to 96 hours on 25mL LB agar plates made with concentrations as stated above. The strains' temperature sensitive phenotype was confirmed upon observing no growth at temperatures above 30°C, after 96 hours.

DNA isolation. All genomic and plasmid DNA extractions were performed using the PureLink Genomic Mini DNA kit (Invitrogen) and the PureLink Quick Plasmid Miniprep kit (Invitrogen) respectively. Genomic DNA from WJKC-1 and WJKC-2 strains was extracted and used in PCR experiments to confirm the chromosomal insertion of the *cam*R cassette. Genomic DNA from BL21(DE3) and plasmid DNA from *E. coli* DH5α strain containing pkD3 plasmid was also isolated as positive controls in PCR experiments.

Primer design. Forward and reverse primers were designed to bind to the downstream region of the *yid*D gene, upstream and downstream region of the *cam*R cassette, and upstream region of the *mnm*E gene (Fig.1A). Primers were designed using the A plasmid Editor (ApE) software. Primer design specifications are described in Table 1. Different combinations of the primers were

included in PCR reactions to amplify the *cam*R cassette, its flanking regions, and the region in between *yid*D *and mnm*E (Fig. 1A). Each PCR reaction was done in a 50 µl mixture containing 1X PCR buffer (-Mg), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of forward and reverse primer, 3 ng/reaction template DNA, 2 U/reaction Platinum *Taq* DNA polymerase (Invitrogen) and DNAse-free deionized water, as indicated by the manufacturer's protocol.

TABLE 1 Sequence, GC content, melting temperature, and length of primers used in PCR

Primer	Sequence (5' - 3')	GC content (%)	Melt-ing Temp-erature (°C)	Length (bp)
yidD-binding [yidDF]	5' – GGGTCCTGATAGCCCTCATTCG – 3'	59	60	22
camR-binding (upstream, external) [CamRUpR]	5' – GAATACCACGACGATTTCCGGC – 3'	55	59	22
camR-binding (downstream, external) [CamRDownF]	5' – CGGATGAGCATTCATCAGGCG – 3'	57	60	21
mnmE-binding [mnmER]	5' – CGGAAGATAATCGGCGTAGCGC – 3'	59	61	22
camR-binding (upstream, internal) [IntCamRF]	5'-CCGCCATTCAGAGTTTAGGAC-3'	52	57	21
camR-binding (downstream, internal) [IntCamRR]	5'-CTGCGTGATGAACTTGAATTGC-3'	45	57	22

Polymerase chain reaction to test primer specificity. Gradient PCR was used with primer pairs IntCamRF and IntCamRR on template pKD3, CamRUpR and CamRDownF on template pKD3, and yidDF and mnmER on genomic DNA template isolated from BL21. Conditions consisted of initial denaturation at 95°C for 3 minutes, 35 cycles of denaturing double-stranded template DNA at 95°C for 30 seconds, primer annealing at a gradient of 58°C to 62°C for 30 seconds and extension at 72°C for 1 minute/Kilo bases, and then a final extension at 72°C for 10 minutes.

PCR to test for *camR*-cassette insertion in WJKC-1 and WJKC-2. As depicted in Figure 1 B and C, the following primer combinations were used to locate *camR*-cassette: yidDF and CamRupR, yidDF and CamRdownF, mnmER and CamRDownF, mnmER and CamRUpR. Primer combinations of IntCamRF and IntCamRR on pKD3 (Fig. 1D), and yidDF and mnmER on BL21 (Fig. 1E) were used as positive controls. The thermocycler conditions mentioned previously were also utilized in this confirmation PCR, except primer annealing was set to 61°C for 30 seconds.

Gel Electrophoresis of PCR amplicons. PCR reaction were resolved at 120 volts for 45 minutes, on a 1% agarose gel stained with SYBR© Safe, in 1x TAE buffer (40 mM Tris, 20 mM Acetate, 1 mM EDTA; pH at 8.6). Bands on the gel were visualized under UV light using AlphaImager® software. PCR mixtures that resulted in bands were purified using the PureLink PCR Purification Kit (Invitrogen).

Ribosomal 16S rRNA gene amplification of WJKC-1 and WJKC-2 genomic DNA. Universal primers 926R and 515F-Y described by Parada *et al.* were used to amplify the region of 16S rRNA sequence approximately 400 base pairs (4). Gradient PCR from 44°C to 56°C on WJKC-1, WJKC-2, and *E. coli* BL21 (DE3) genomic DNA template was performed using the Platinum *Taq* DNA Polymerase (Invitrogen) protocol. Amplicons from the 50°C annealing temperature were purified using the PureLink PCR Purification Kit (Invitrogen). Bovine Serum Albumin was added to WJKC-1 sample at 50°C annealing temperature to stabilize the primers as outline by Parada *et al.* (5).

Sanger sequencing and analysis of PCR products. All Sanger sequencing was performed by the NAPS unit at the University of British Columbia. Sample preparation included dilution of the PCR products to 15 ng/µl and the primers to 10 pmol/µl. The samples corresponding to these sequencing results were identified by Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information website.

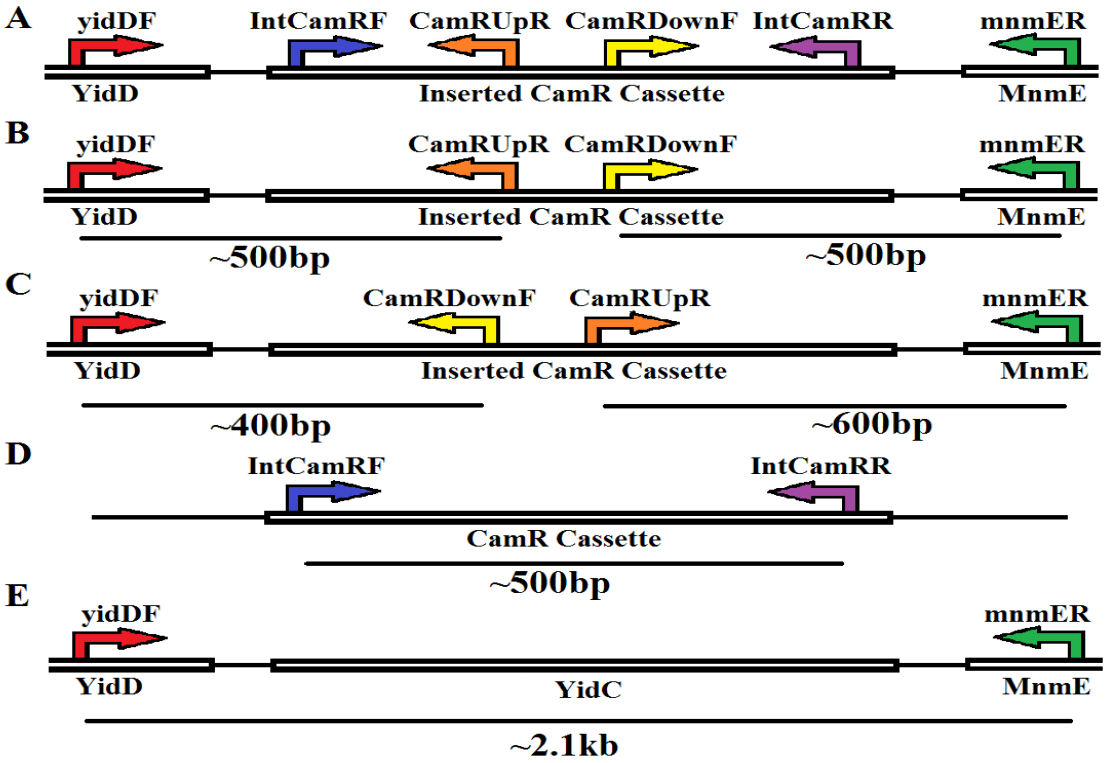

FIG. 1 Primer-binding regions on WJKC-1 and WJKC-2 template DNA, primer combinations used in PCR and the respective expected sizes. (A) Primer design for yidD, camR-cassette, and mnmE. Primer combinations: **(B)** yidDF-CamRupR and CamRdownF-mnmER. **(C)** yidDF-CamRDownF and CamRUpR-mnmER. **(D)** IntCamRF-IntCamRR for the amplification of the ~500bp *camR*-cassette region on pkD3 plasmid. **(E)** yidDF-mnmER for the amplification of the 2.1kb region in *E. coli* BL21.

RESULTS

Confirmation that strains WJKC-1 and WJKC-2 grow at 20°C and 30°C but not at 37°C. Choi *et al.* observed growth of WJKC-1 and WJKC-2 on LB media supplemented with kanamycin and chloramphenicol at 20°C and 30°C but not at 37°C (1). We began our study by repeating these growth experiments. On plates, colony forming units appeared after 48 to 72 hours of incubation at 30°C. The colonies were raised, circular with 1 to 2 mm in diameter, with entire margins, and a shiny and smooth appearance. In liquid culture media became turbid after 48 to 72 hours of shaking at 30°C. Both strains grew in the presence of chloramphenicol and kanamycin. Both WJKC-1 and WJKC-2 grew at 20°C and 30°C after 48 to 72 hours, but there were no visible colonies on the plates incubated at 37°C after 98 hours. Green fluorescence was observed at the poles of the cells using ultraviolet light microscopy, an observation consistent with the results of Choi *et al*. Taken together these results show that WJKC-1 and WJKC-2 are resistant to chloramphenicol and kanamycin, showed temperature sensitive growth below 37°C, and green fluorescence which consistent with the expected phenotype of a yidC knockout strain of E. coli complemented with a *yidC* variant fused to GFP (1).

PCR of WJKC-1 and WJKC-2 genomic DNA yields amplicons of unexpected size. We designed a PCR based approach to confirm the presence and proper location of the *camR* cassette. The locations at which the primers bind to the genomic DNA are outlined in Figure 1A. To ensure proper primer binding, primers of yidDF and mnmER up and down stream of yidC were used to amplify a 2.1kb region in BL21. Primers of IntCamRF and IntCamRR within the CamR cassette were used to amplify a 527bp region of pKD3. Primers of CamRdownF and CamRupR facing out were used to amplify the rest of 2.7kb region in pKD3. All primers were found to bind and amplify a single region, all of them are of expected size except for the combination of CamRdownF and CamRupR, which yielded a >5kb band. The extensive elongation time might have led to circular amplification on pKD3 plasmid beyond the desired template region, resulting in an amplicon twice the size of the plasmid itself.

We amplified *camR-mnmE* and *camR-yidD* flanking regions to provide a definitive answer about the location of *camR* in relation to *yidD* and *mnmE* (Fig. 2A). Positive controls for this reaction showed bands of the expected size: ~500bp for CamR cassette in pKD3, and ~2kb for *yid*D to *mnm*E in BL21(DE3) (Fig. 2B). Amplification of the putative regions from *yid*D to *cam*R and *cam*R to *mnm*E showed bands of unexpected sizes. As shown in Figure 2A, lanes 1 and 2, PCR amplifications with yidDF and CamRUpR primers resulted in a ~1.5kb amplicon. This is different from the expected size of ~300bp. PCR amplification using CamRDownF and mnmER primers yielded a ~750bp band, which is also different from the

expected size of ~400bp (lanes 5 and 6). These amplicons may have been a result of non-specific binding of primers to the template. There was no amplification using yidDF with CamRdownF or mnmER with CamRUpR (lanes 3, 4, 7, and 8). The ~750bp *camR-mnmE* amplicon from WJKC-1 was sent for sequencing to identify the amplified region.

Sequence of the ~750bp *camR-mnmE* amplicon in WJKC-1 show a 90% sequence alignment with a *Rhizobium Tropici* CIAT 899c plasmid. Sequencing results were obtained from the ~750bp *camR-mnmE* amplicon (Fig. 2A lanes 5 and 6). The sequence of amplicon had a 90% sequence alignment with *Rhizobium tropici* CIAT 899 when subjected to BLAST. 697bp of the amplicon was sequenced with no interfering peaks. We requested the amplicon to be re-sequenced, but we obtained the same sequence alignment. Based on this result, we decided to investigate the species identity of WJKC-1 and WJKC-2.

Previous literature and ribosomal 16S rRNA sequencing confirms WJKC-1 and WJKC-2 as *Rhizobium* species. We hypothesized that the 30°C temperature sensitive phenotype in WJKC-1 and WJKC-2 is observed because they belong to a subset class of *Rhizobium.* Previous studies have shown that some subsets of *Rhizobium* bacteria have an optimal temperature of 29°C to 31°C, but are not viable at 37°C (4). In our attempt to grow WJKC-1 and WJKC-2, we observed a similar temperature-sensitive phenotype. The study also reported that many subsets of *Rhizobium* show visible growth at 48 to 72 hours post-inoculation (4), similar to what we observed in our study. Fluorescence microscopy showed WJKC-1 and WJKC-2 to be rod-shaped, which is consistent with *Rhizobium* morphology (4).

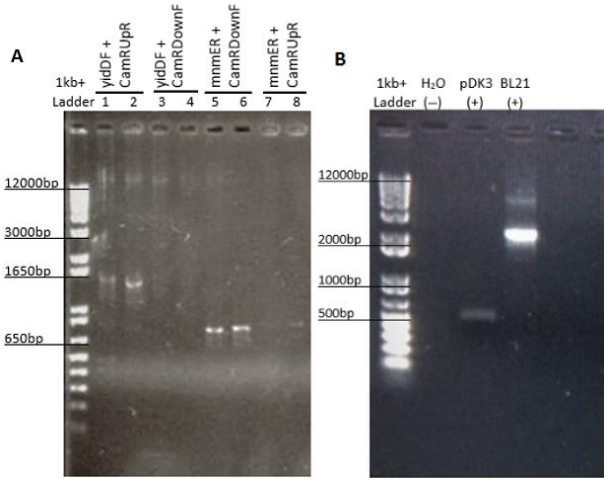

FIG. 2 Agarose gel electrophoresis analysis of PCR products from *camR*-cassette junction in WJKC-1 and WJKC-2 genomic DNA, and controls using water, pKD3, and BL21. (A) Amplification using yidDF and CamRupR primers in WJKC-1 (lane 1) and WJKC-2 (lane 2) resulted in >1650bp bands. Amplifcation using mnmER and CamRDownF primer in WJKC-1 (lane 5) and WJKC-2 (lane 6) resulted in ~650bp bands. Amplification using YidDF and CamRdownF in WJKC-1 (lane 3) and WJKC-2 (lane 4) resulted in no bands. Amplification using mnmER and CamRupR in WJKC-1 (lane 7) and WJKC-2 (lane 8) also resulted in no bands. **(B)** PCR amplification of pKD3 using IntCamRF and IntCamRR resulted in a ~600bp band, which is expected. PCR amplification of E. coli BL21 using yidDF and mnmER resulted in a ~2kb band, which is also expected. Negative control did not show bands.

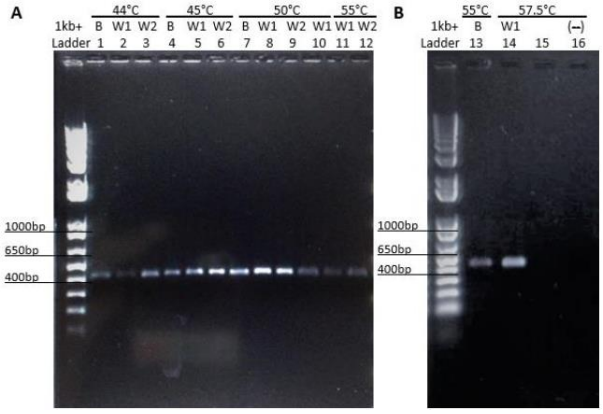

FIG. 3 Agarose gel electrophoresis of 16s rRNA amplification of WJKC-1 (W1), WJKC-2 (W2), and E. coli BL21 (B) positive control by gradient PCR at 44°C, 45°C, 50°C, 55°C, 57°C. PCR amplification using 926R and 515F-Y primers resulted in a ~400bp product at all annealing temperatures.

We investigated the species of WJKC-1 and WJKC-2 by performing 16S rRNA PCR via 926R and 515F-Y 16S universal primers to amplify a ~400bp sequence of 16S rRNA (Fig. 3) (5). Sequencing and genetic analysis of the ~400bp product showed 100% sequence-similarity with *Rhizobium leguminosarum*, *Rhizobium Tropici*, and multiple experimentally modified strains of *Rhizobium* (Fig. S1). We also sequenced the ~400bp amplicon from *E. coli* BL21 as a positive control, which resulted in a 100% sequence-similarity with *E. coli.* Analysis of chromatogram from Sanger sequencing showed no interfering peaks, suggesting that there were no unwanted sequences that could have resulted in a false positive for *Rhizobium.* Identification of WJKC-1 and WJKC-2 as *Rhizobium*-derived strains may suggest the amplification of ~750bp bands upon using yidDF-CamRUpR and mnmER-CamRDownF primers is the result of nonspecific binding.

DISCUSSION

Our study initially characterized WJKC-1 and WJKC-2 strains, specifically focusing on the possibility of induction of polar effects by the insertion of *camR* cassette, yielding a temperature sensitive phenotype. In the previous study by Choi *et al.*, these putative *E. coli* strains showed growth 48 hours after inoculation at 20°C and 30°C but not at 37°C (1). In our attempt to locate the *camR*-cassette in WJKC-1 and WJKC-2 by PCR, the *camR-mnmE* junction sequence had 90% sequence similarity with a *Rhizobium Tropici* CIAT899c plasmid. This result led to the investigation of the species of WJKC-1 and WJKC-2 using 16s rRNA sequencing. 16s rRNA sequencing results confirmed that WJKC-1 and WJKC-2 are strains of *Rhizobium.*

We were unable to identify the specific subclass of *Rhizobium* from which WJKC-1 and WJKC-2 may have

been derived because of the limitations of 16s rRNA sequencing. We expected that the subclasses of *Rhizobium* would have identical 16S rRNA sequences. Evidently, 16s rRNA sequencing showed a 100% sequence match with *R. legiminosarum, R. Tropici,* and multiple genetically modified strains of *Rhizobium* (Fig. S1). It is possible that WJKC-1 and WJKC-2 could belong to *R. legiminosarum* strain, because it is documented to have optimal temperature growth from 27°C-29°C (6). In contrast, there is emerging evidence that *R. tropici* can tolerate temperatures above 35°C, which is not observed with WJKC-1 and WJKC-2 (4). However, further extensive genomic analysis of WJKC-1 and WJKC-2 would be needed to identify the specific subclass.

According to Choi *et al.,* pEHI-*yid*C-*GFP* was transformed into the bacterial strain before knocking out genomic *yid*C via lambda-red recombination system. Consequently, the group observed under UV microscope that the transformants showed green fluorescence. In our study, we also observed GFP expression at the poles of the cell. This suggests that *Rhizobium* contamination likely occurred before pEHI-*yid*C-*GFP* insertion into the host bacterium. This also suggests that *Rhizobium* contamination did not occur during the experimental procedures in our study. This reasoning is consistent with why Choi *et al.* observed the 30°C temperature-sensitive phenotype.

We speculate that during construction of WJKC-1 and WJKC-2 strains, Choi *et al.* used growth conditions that promoted *Rhizobium* growth and contamination. The lambda-red recombination system is encoded on a pKD46 plasmid that functions at temperatures 30°C and below (7). Consequently, Choi *et al.* incubated the transformants at 30°C to ensure pKD46 survival prior to genomic *yid*C knockout. The 30°C temperature is suitable for growth of some *Rhizobium* species (2). Choi *et al.* selected for transformants by incubating the *yid*C knockout strains in LB-ampicillin, chloramphenicol, and kanamycin. The *Rhizobium* contaminants were able to survive selection by these antibiotics since they had also been transformed with the pKD46, and pKD3 that contains ampicillin and kanamycin resistance genes, respectively. Additionally, some subclasses of *Rhizobium* encode chloramphenicol resistance genes that are not sequence similar to pKD3-derived chloramphenicol resistance. Choi *et al.* also observed WJKC-1 and WJKC-2 morphology under fluorescence microscopy to verify strain construction. However, since *E. coli and Rhizobium* both are rod-shaped Gram-negative bacteria, *Rhizobium* contamination would not have been evident by microscopic observations**.**

The procedure used by Choi *et al.* to construct WJKC-1 and WJKC-2 reflects the challenges in generating a *yidC* knockout strain. *yidC* is an essential gene in *E. coli* that assists in the folding and insertion of membrane proteins (8); a knockout mutation would be lethal to the cell. The cells must first be complemented with an alternative source of the *yidC* before the chromosomal knockout for the strain to survive. Additionally, the usage of Lambda-red recombination system may lead to the disruption of expression of adjacent genes by polar effects or the non-specific knockout of other genes.

WJKC-1 and WJKC-2 were constructed to study the role of YidC P1 domain in *E. coli* (1). Here we have shown using 16s rRNA sequencing that WJKC-1 and WJKC-2 are strains of a subclass of *Rhizobium* that grows at temperatures 30ºC and below. *Rhizobium* contamination can occur if growth or temperature conditions that favor *Rhizobium* survival are used during the knockout procedure. Using a different method other than Lambda-red recombination system to excise the chromosomal *yidC* would prevent growth at 30°C or below, which favour *Rhizobium* growth.

FUTURE DIRECTIONS

Since *Rhizobium* is considered to be a non-model organism, one can re-attempt to construct a *yid*C chromosomal knockout strain in *E. coli* by alternative techniques other than lambda-red recombination system. Alternative methods would eliminate the need for 30°C incubation, which favours *Rhizobium* survival. One such method could be using Homology Directed Repair by CRISPR/Cas9 to replace the chromosomal *yidC* with *yidC-GFP or yidC-P1-GFP*. Homology Directed Repair would be mediated by the insertion of a template DNA that encodes *yidC-*Δ*p1-GFP*. The GFP fused to YidC serves as an indicator that the chromosomal *yidC* has been knocked out.

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