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Bacterial Genome Editing Using CRISPR/Cas9 Coupled with Lambda Red Recombineering for Potential Generation of an Antigen 43 Knockout

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SUMMARY To study the effect of differential Antigen 43 expression on biofilm formation, we attempted to generate a knockout mutant of the *flu* gene in a well-characterized *Escherichia coli* lab strain, MG1655. CRISPR/Cas9 technology was used, in tandem with lambda Red recombination machinery to assist with homologous recombination. A pCasRed plasmid expresses the Cas9 endonuclease, trans-activating CRISPR RNA, and lambda Red machinery. A pCRISPR-SacB-gDNA plasmid was successfully constructed to express guide RNA, allowing targeted cleavage by the Cas9 enzyme at the *flu* gene. Double stranded donor DNA oligonucleotides were designed as a repair template in recombineering to remove the protospacer and protospacer adjacent motif sequence, while inserting an in-frame stop codon at the target locus. Mutagenesis was attempted by heat shock transformation with pCasRed, pCRISPR-SacB-gDNA, and donor DNA. After screening by colony PCR and sequencing of the PCR products, a *flu* knockout mutant could not be identified. In addition, high levels of unedited background escaper colonies were observed. We propose that improved guide RNA targeting sites, the use of longer donor DNA, and transformation by electroporation may improve CRISPR/Cas9 mutagenesis efficiency.

INTRODUCTION

Antigen 43 (Ag43) is a surface-displayed autotransporter protein that mediates autoaggregation of *Escherichia coli* through intercellular Ag43-Ag43 interactions (1). The cell-to-cell interaction by Ag43 has a role in mediating biofilm formation in stress conditions, as reduced Ag43 expression is associated with lower biofilm formation (2, 3). The generation of a *flu* knockout would help determine if Ag43 is essential for the formation of biofilm, and would be a useful model for further study of this surface protein in *E. coli*. Previous attempts for a *flu* gene knockout mutant strain have been made (1, 4, 5), including one from the Keio collection, which was determined not to be a *flu* knockout by the Coli Genetic Stock Center (CGSC). However, a *flu* gene knockout for the lab strain, *E. coli* MG1655, has not been generated.

The CRISPR/Cas9 editing system is a useful tool for genome editing due to its high target specificity using a small RNA, its wide choice of targets upstream of an NGG protospacer adjacent motif (PAM), and its potentially high efficacy when used with recombineering (6). The *Streptococcus pyogenes* Cas9 endonuclease, with a bound trans-activating crRNA (tracrRNA) and guide RNA (gRNA), is directed to a complementary protospacer sequence upstream of an NGG PAM site (6). This results in cleavage of the target locus DNA and a double-stranded break, which is lethal to the bacteria (7, 8). Repair of the DNA can be performed using a lambda Red system to facilitate recombineering with a supplied donor DNA (dDNA) repair template (7). Mutagenesis can occur when bacterial strains harbor plasmids expressing the CRISPR/Cas9 system and lambda Red machinery with a dDNA repair template. A pCasRed plasmid expresses Cas9, tracrRNA, and lambda Red genes and a pCRISPR-SacB-gDNA plasmid expresses gRNA (9). CRISPR/Cas9 genome editing is limited by the background unedited 'escaper' colonies, which are able to evade cleavage by Cas9, usually due to mutations in the PAM or the protospacer sequences (6). With high efficiency recombineering, the number of escaper colonies is expected to be low. The adaptation of the CRISPR/Cas9 system for efficient bacterial genomic editing makes it an appealing method for mutagenesis. As a result, we attempted to use CRISPR/Cas9 to generate a *flu* knockout mutant in this study.

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TABLE 1. Lists of plasmids and *Escherichia coli* strains used

Plasmid or Strain	Relevant characteristics[a]	Reference
Plasmid		
pCasRed	Derived from the pCas9 plasmid, expresses Cas9 nuclease and the trans-activating CRISPR RNA (tracrRNA) constitutively, expresses λ Red (Exo, Beta, Gam) cassette using arabinose-inducible promoter (pBAD), Cam^R	(9)
pCRISPR-SacB	Derived from the pCRISPR plasmid and the pKM154 plasmid, BsaI cloning site for gDNA, Kan^R gene fused to *Bacillus subtilis* levansucrase (*sacB*)	(9)
pCRISPR-SacB-gDNA	Expresses guide RNA (gRNA) constitutively using PLtetO-1 promoter, Kan^R gene fused to *Bacillus subtilis* levansucrase (*sacB*)	This Study
Strain		
DH5α	F^- *λ⁻ recA1 Δ(argF-lac)169* φ80d*lacZ58*(M15) *gyrA96*(NalR) *glnV44*(AS) *rfbC1 ΔphoA8 deoR481 endA1 hsdR17 thi-1*	CGSC #12384
MG1655	F^- *λ⁻ rph-1*	CGSC #6300

[a]Cam^R, Chloramphenicol resistance; Kan^R, Kanamycin resistance

METHODS AND MATERIALS

Bacterial strains and growth conditions. Descriptions of the pCasRed, pCRISPR-SacB, and pCRISPR-SacB-gDNA plasmids used in this study are found in Table 1. The genotype of *E. coli* DH5α and MG1655 bacterial strains used are also found in Table 1. *E. coli* DH5α was used for construction of the gRNA expressing pCRISPR-SacB-gDNA plasmid and *E. coli* MG1655 was used for mutagenesis at the *flu* locus. *E. coli* grown in liquid lysogeny broth (LB) media (1% tryptone, 0.5% yeast extract, and 1% NaCl in distilled water) were incubated at 37°C, shaking at 200 rpm. *E. coli* strains grown on solid LB agar (1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% Select agar in distilled water) plates were incubated stationary at 37°C. LB broth and agar cultures containing antibiotics had the following concentrations: kanamycin at 50 µg/mL and chloramphenicol at 25 µg/mL.

Preparation of $CaCl_2$ competent cells and heat shock transformation. Competent cells for heat shock transformation was done using the Hancock Lab protocol (10), but modified to allow for long term storage at -80°C. Overnight culture (MG1655 or DH5α) was diluted 1:200 into fresh LB (the volume of which is used for subsequent steps) and incubated at 37°C, shaking at 200 rpm. After the culture reached an OD_{550} between 0.2 to 0.4, the culture was placed on ice for 10 minutes. The cells were centrifuged at 10,000 rpm and 4°C for 5 minutes, resuspended in half culture volume of 0.1 M $CaCl_2$, and left on ice for 25 minutes. The cells were centrifuged at 10,000 rpm and 4°C for 5 minutes, resuspended in 1/50 culture volume of 0.1 M $CaCl_2$ with 10% glycerol, and transferred into aliquots in Eppendorf tubes. Competent cells were then stored in the -80°C freezer. Prior to heat shock transformation, 100 µL of pre-thawed competent cells were mixed with DNA gently by swirling. Competent cells were heatshocked at 42°C for 45 seconds and incubated on ice for 2 minutes. LB was added to transformed cells and allowed to recover at 37°C, shaking at 200 rpm for 1 hour. The cells were plated on LB agar plates with corresponding antibiotics and incubated overnight at 37°C.

Construction of the pCRISPR-SacB-gDNA plasmid. The design and construction of the pCRISPR-SacB-gDNA plasmid was based on previous literature (11). Guide DNA (gDNA) oligonucleotides were designed based on a 30 bp protospacer region upstream of a NGG PAM site with overhanging nucleotides to allow for ligation into BsaI digested pCRISPR-SacB, after annealing. The sequences of gDNA oligonucleotides used are listed in Table 2 and the targeted PAM and protospacer is found in Figure S1. Complementary gDNA oligonucleotides

were phosphorylated with T4 Polynucleotide Kinase (ThermoFisher) at 37°C for 30 minutes. NaCl was added to the reaction, which was subsequently heated to 95°C for 5 minutes and annealed by allowing cooling to 25°C by decreasing increments of 0.1°C every minute using a thermocycler. 2 µg of pCRISPR-SacB was digested with BsaI (New England Biolabs) at 37°C for 1 hour. Digested pCRISPR-SacB was treated with the PureLink PCR Purification Kit (ThermoFisher) and 500 ng of cleaned product was dephosphorylated with calf intestinal alkaline phosphatase (ThermoFisher) at 37°C for 2 hours. The reaction was separated using 1% agarose gel electrophoresis and the large fragment (about 3.5 kb expected size) was excised and isolated using the PureLink Quick Gel Extraction Kit (ThermoFisher). 30 ng of the cut and dephosphorylated pCRISPR-SacB and phosphorylated, annealed gDNA was ligated with T4 DNA ligase (ThermoFisher) at 16°C overnight. The ligated plasmid was transformed into DH5α competent cells. Plasmid isolation was conducted from colonies using the PureLink Quick Plasmid Miniprep Kit (ThermoFisher) and verified by sequencing with GENEWIZ, using the Neo-Rev primer (Table 1).

CRISPR/Cas9 mutagenesis of MG1655 at the flu locus. The bacterial CRISPR/Cas9 mutagenesis was done as previously described (11), but with use of a 60 bp linear dDNA and varying arabinose treatments. The 60 bp double stranded dDNA was designed to delete the 30 bp protospacer and the PAM site, replacing them with an in-frame TAA stop codon. The sequences of the dDNA Oligo I and II are found in Table 2 and the design for flanking homologous arms is found in Figure S1. Aliquots containing 10 µg of dDNA Oligo I and 10 µg of dDNA Oligo II were heated at 95°C for 5 minutes and allowed to anneal by cooling to 25°C at -0.1°C/minute using a thermocycler. Confirmation of dDNA annealing was done using 2% agarose gel electrophoresis. MG1655 competent cells containing pCasRed, prepared with incubation with LB chloramphenicol and 0.2% arabinose from overnight culture in LB chloramphenicol only, were heatshocked with 100 ng pCRISPR-SacB-gDNA and 10 µg annealed dDNA. The cells were allowed to recover in LB with 0.2% arabinose. The cells were subsequently plated on LB agar plates containing 0.2% arabinose, chloramphenicol, and kanamycin.

Colony PCR (cPCR) of double transformants for confirmation of flu knockout. Colonies transformed with pCasRed and pCRISPR-SacB-gDNA for screening were streaked onto a fresh LB agar plate containing chloramphenicol and kanamycin and incubated overnight at 37°C. Using the same pipette tip for streaking, residual bacteria were suspended into a PCR

TABLE 2. Oligonucleotides and primers used in bacterial CRISPR/Cas9 mutagenesis.

Primer[a] or oligonucleotide	Sequence (5′ – 3′)	T_m (˚C)	% GC	Length (nucleotides)
gDNA Oligo I	AAACCTGCTACAGGCTGGTATGGAATCACATGACG	66.4	48.6	35
gDNA Oligo II	AAAACGTCATGTGATTCCATACCAGCCTGTAGCAG	64.9	45.7	35
dDNA Oligo I	GAAAAGCTGATGAAACGACATCTGAATACCTAACGCTTTCGTGGTTGCCTCCGAACTGGC	72.3	48.3	60
dDNA Oligo II	GCCAGTTCGGAGGCAACCACGAAAGCGTTAGGTATTCAGATGTCGTTTCATCAGCTTTTC	72.3	48.3	60
Fwd cPCR Primer	CGGTATCCACGTTTGTGGGTAC	59.2	54.5	22
Rev cPCR Primer	TGACGTGACTGCGGCAAG	60.1	58.4	18
Neo-Rev Sequencing Primer	GCCCAGTCATAGCCGAATAG	56.5	55.0	20

[a]Fwd, Forward; Rev, Reverse. Sequence analytics from IDT Biophysics and OligoAnalyzer.

mix with primers listed in Table 2. PCR reaction mixtures were incubated in a thermocycler at 95°C for 5 minutes, 35 cycles of 95°C, 55°C, and 72°C for 30 seconds at each temperature, 72°C for 5 minutes, and a final holding temperature of 4°C. PCR reaction mixtures were separated using 2% agarose gel electrophoresis, with the wild-type colony and *flu* knockout colony PCR products expected to be 200 bp and 170 bp in size respectively. Selected PCR products were sent for sequencing by GENEWIZ, using the Rev cPCR primer (Table 2), after treatment with the PureLink PCR Purification Kit (ThermoFisher).

Assessment of gRNA targeting efficiency with CHOPCHOP v2. Target sequences by gRNA for Cas9 cleavage was identified using CHOPCHOP v2 (12), for the *flu* target in *E. coli* (str. K-12/MG1655) using CRISPR/Cas9. A 30 length sgRNA without PAM was used, but all other parameters were at the default setting. NGG PAM sites were identified with up to 3 mismatches in the protospacer for off-targets in the genome. The Xu *et al.* metric only for NGG PAM was used for the efficiency score (13).

RESULTS

A pCRISPR-SacB-gDNA plasmid was successfully constructed. To express gRNA for targeted cleavage of the *flu* locus by pCas9, the pCRISPR-SacB-gDNA plasmid was constructed to be transformed into MG1655 for mutagenesis. Construction of the plasmid was conducted by ligating pCRISPR-SacB, cut with BsaI, with annealed gDNA oligonucleotides. Transformation of the ligation reaction yielded 10 colony forming units and transformation with BsaI digested pCRISPR-SacB only, yielded 3 colonies. Of the plasmids sequenced from four colonies, only one showed successful cloning of the pCRISPR-SacB-gDNA with the gDNA sequence flanked by direct repeat (DR) sequences and the loss of the BsaI restriction enzyme cut sites (Fig. S2A). The other plasmids that failed to contain the cloned gDNA sequence were found to retain one copy of the direct repeat sequence and to have lost the BsaI restriction enzyme cut sites (Fig. S2B), suggesting that the plasmids self-ligated rather than failed to be cut by BsaI. Correct construction of pCRISPR-SacB-gDNA suggests that it was suitable for expression of gRNA.

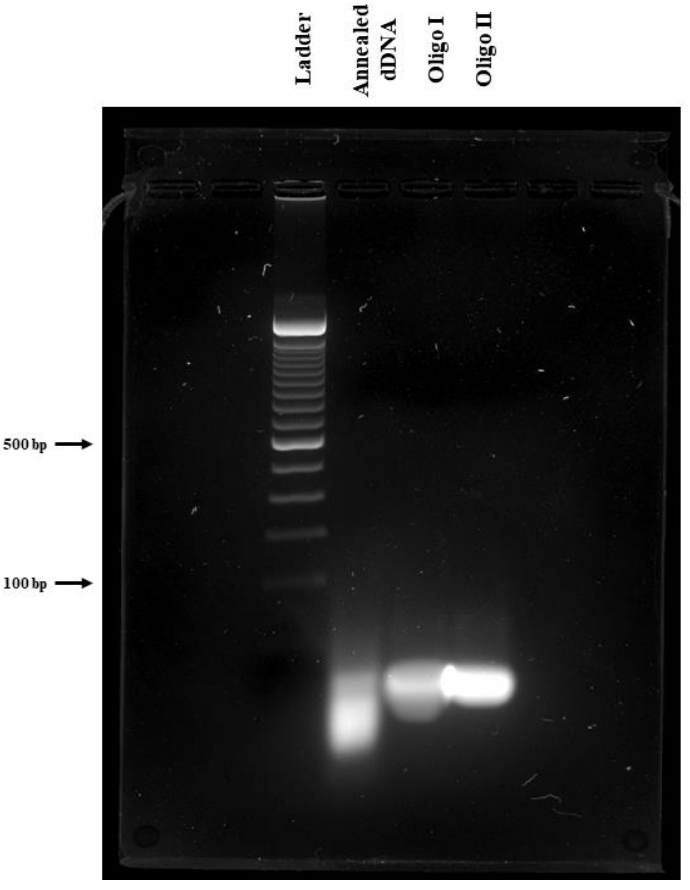

FIG. 1 Gel electrophoresis of dDNA. Annealed double stranded dDNA (10 µg) and single stranded Oligo I (10 µg) and Oligo II (10 µg) run on a 2% agarose gel. Double stranded and single stranded dDNA are expected to be 60 bp long.

Donor DNA was confirmed to be annealed. To ensure that the linear donor DNA (dDNA) was annealed as a double stranded repair template for mutagenesis, 2% agarose gel electrophoresis was used to compare with single stranded dDNA oligonucleotides. Annealed dDNA exhibited a different band pattern than that of either Oligo I or Oligo II as shown in Figure 1. The double stranded and single stranded dDNA bands were not sharp, but had strong signal intensity with loading of 10 µg for each treatment. The 60 bp bands were at the expected size, reasonably below the 100 bp marker of the DNA ladder. Proper annealing of the dDNA suggests that it was suitable for transformation in mutagenesis.

Attempted mutagenesis of the *flu* gene by CRISPR/Cas9. For mutagenesis at the *flu* locus, MG1655 was transformed with pCasRed and pCRISPR-SacB-gDNA, supplied with linear DNA as a repair template. MG1655 was also transformed without donor DNA as a control for background unedited cells or escapers. With the use of arabinose induction for lambda Red machinery expression, attempted mutagenesis yielded about 70 colony forming units (Fig. 2A) and about 50 colony forming units without donor DNA (Fig. 2B) when plated on chloramphenicol and kanamycin plates with 0.2% arabinose. However, when plated without arabinose, attempted mutagenesis yielded about 50 colony forming units (Fig. 2C) and about

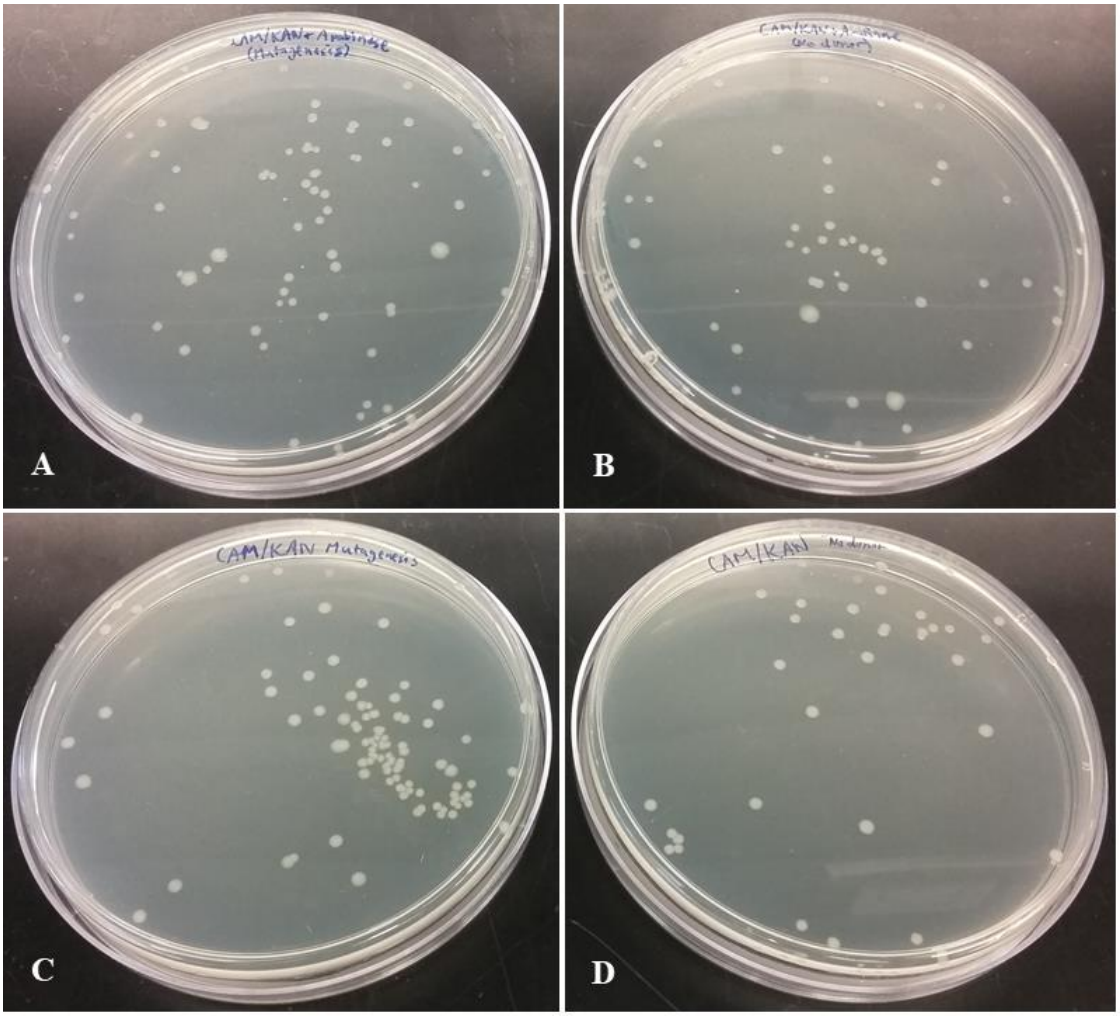

FIG. 2 Plated double-transformants of pCasRed and pCRISPR-SacB-gDNA after heat shock transformation. (A, C) Colony forming units from cells transformed with pCasRed, pCRISPR-SacB-gDNA, and dDNA. (B, D) Colony forming units from cells transformed with pCasRed and pCRISPR-SacB-gDNA, without dDNA. Transformants were plated on agar plates containing chloramphenicol and kanamycin with 0.2% arabinose in A and B and without arabinose in C and D. There were approximately 70 and 50 colony forming units on A and B respectively and 50 and 30 colony forming units on C and D respectively.

30 colony forming units without donor DNA (Fig. 2D). Earlier experiments without any arabinose induction yielded approximately 20-40 colonies and 20 colonies for attempted mutagenesis without donor DNA respectively. Extended induction of arabinose may be associated with a higher number of transformants, as it was highest with arabinose recovery and using arabinose plates, and lowest without any arabinose. Overall, these findings suggest that there was a high level of escapers that would affect selection of a *flu* knockout mutant.

Arabinose may affect the size of transformed colonies. With induction of arabinose, colony diameters ranged from less than 1 mm to 4 mm when plated with arabinose. However, when plated without arabinose, colony size was more consistent between 2 mm to 3 mm. All colonies without any use of arabinose were about 1 mm in diameter or less. This suggests that the use of arabinose at different stages of mutagenesis had an effect on colony morphology.

Arabinose induction is required for colony transfer survival. To retain potential strains screened by colony PCR, picked colonies were transferred to a fresh plate prior to mixing into PCR reaction tubes. All colonies streaked from double-transformed competent cells with arabinose induction were able to grow on chloramphenicol and kanamycin plates, without arabinose (Fig. 3). However, without any arabinose induction, several attempts of re-plating colonies on chloramphenicol and kanamycin were not successful. This suggests that initial arabinose induction was required for colonies to survive on fresh antibiotic medium.

***flu* knockout strain was not found by colony PCR.** To verify colonies as potential *flu* knockout isolates, over 50 colonies were screened using colony PCR for a 30 bp deletion. The expected PCR product for wild-type MG1655 and the *flu* knockout is approximately 200 and 170 bp respectively. As a result, any potential *flu* knockout should have a slightly lower band when visualized by gel electrophoresis. As shown in Figure 4, colonies screened had PCR product sizes similar to that of wild-type. Selected PCR products that exhibited slightly lower sizes were sent for sequencing, but all were found to have wild-type *flu* sequences without mutations (Fig. S3). As a result, no *flu* knockout mutants were isolated and identified by colony PCR.

The gRNA targeting efficiency score was determined to be low by CHOPCHOP v2. To determine efficiency of Cas9 cleavage at the *flu* locus for the gDNA sequence used in this study, the CHOPCHOP v2 tool was used (12). No self-complementarity was found, as there were no self-complementary 4-nucleotide stems. There was only one specific target found in

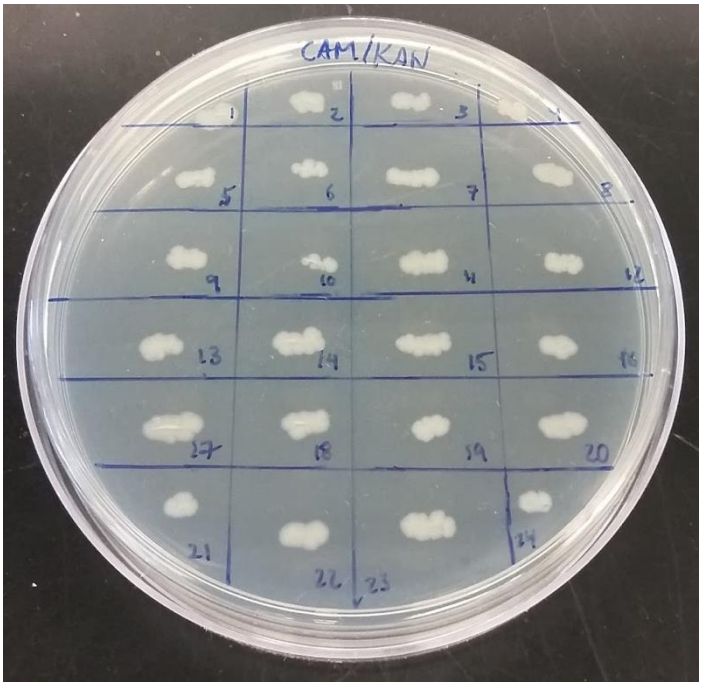

FIG. 3 Transferred colonies from attempted mutagenesis plate. All colonies transferred from double-transformed colonies grew on chloramphenicol and kanamycin plates without arabinose.

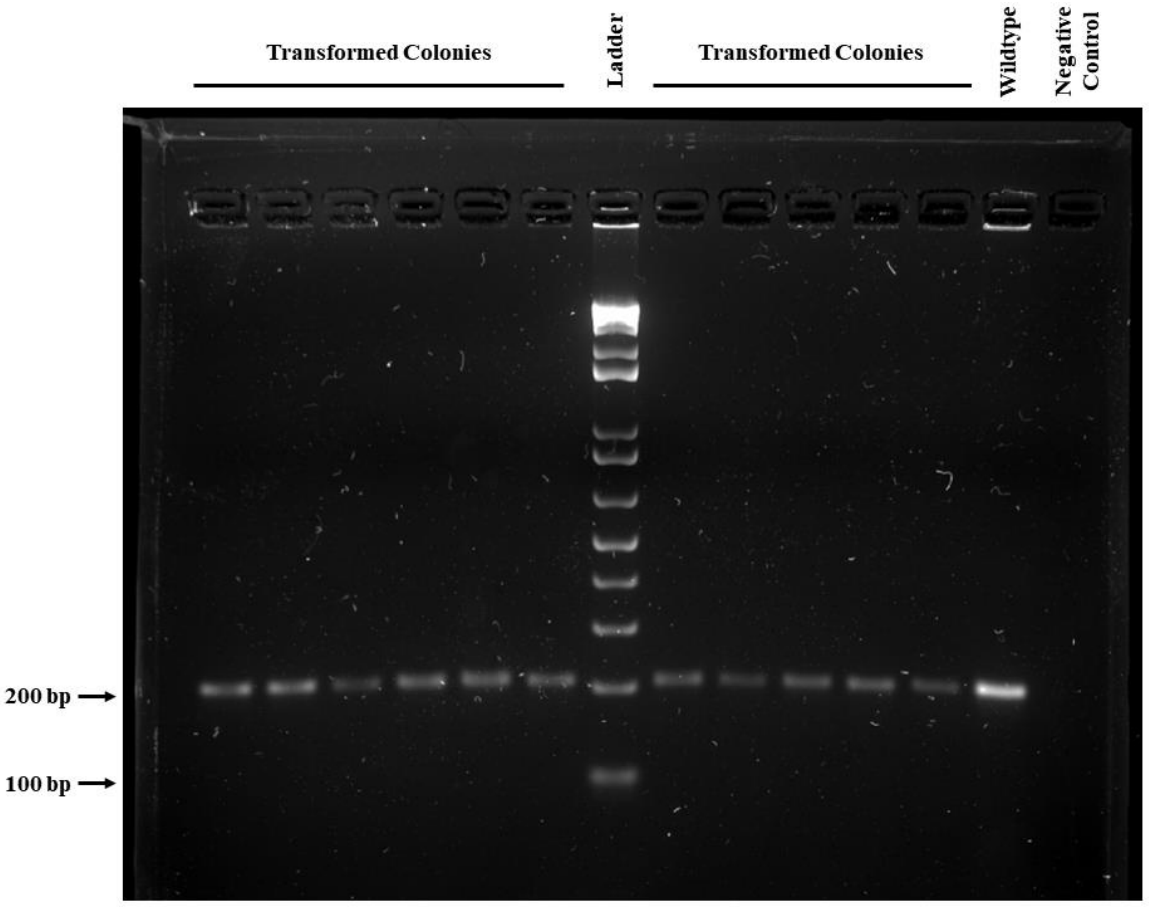

FIG. 4 Representative gel electrophoresis of colony PCR. Colony PCR products of double transformed colonies in mutagenesis run on a 2% agarose gel. A MG1655 colony was used as a control for wild-type and a PCR reaction mix without a picked colony served as a negative control. The PCR products of the transformed colonies had an approximate size of 200 bp, matching that of the wild-type colony.

the *flu* gene for the 30 bp gDNA sequence and there were no off-targets with 1 to 3 mismatches. The calculated gRNA targeting efficiency of the selected site (NC_000913.3:2071559) was 0.47, ranking 331 out of 537 targeting sites. Of the 537 targeting sites, efficiencies ranged from 0.30 to 0.80. As a result, the gDNA sequence used in this study was on the lower end of gRNA targeting efficiency.

DISCUSSION

The initial aim of this study was to generate a *flu* knockout mutant in the well-characterized *E. coli* lab strain MG1655 using CRISPR/Cas9 and lambda Red recombination technologies.

All MG1655 cells transformed with the CRISPR/Cas9 system should have generated a double-stranded break in the *flu* gene, which is lethal and selects against wild-type sequences (8). Homologous recombination with the donor DNA would result in successful repair of the break and generation of the *flu* knockout mutant by creating a 30 bp deletion and an in-frame stop codon insertion. Non-homologous end joining should not be able to rescue *E. coli* cells from repairing CRISPR-mediated double-stranded breaks, as it has been reported to work poorly in *E. coli* (11). Arabinose induction of lambda Red machinery is very important in improving mutagenesis efficiency, however, it may be possible that *E. coli* can repair double stranded breaks with its native *rec* genes or alternative end-joining pathways when supplied with donor DNA (14, 15, 16).

Despite many attempts with colony PCR and sequencing, all colonies screened were determined to be wild-type and were considered escaper colonies. However, it is possible that given the high background of escaper colonies, a knockout mutant colony may be present and

could be found with more screening. Escaper colonies can exist due to spontaneous mutations of Cas9 targets such as the protospacer and PAM sites (17). However, sequencing results of escapers found that the *flu* gene had no mutations, being identical with the wild-type MG1655 *flu* gene sequence, suggesting that survival was due to a different mechanism. It is important to determine the cause of the significant number of escaper colonies observed. If the number of escaper colonies can be minimized, it would greatly save time and resources in performing PCR and sequencing tests to screen for a mutant colony against background wild-type escaper colonies. In addition, the protocol could be optimized to increase mutagenesis efficiency, as some bacterial CRISPR/Cas9 protocols have reported to yield gene editing of *E. coli* with nearly 100% efficiency (17).

Interestingly, a BW25113 *flu* knockout mutant from the Keio collection was listed on the CGSC (4), but has since been removed from the repository. The mutant may have been thought to be generated, but confirmatory analyses rejected that it was a *flu* knockout mutant. It is possible that the *flu* gene has some essential function for *E. coli* survival, outside of Ag43 expression and biofilm formation, which make it difficult for knockout mutants to survive. For this reason, or for some other unexplored cause, there may be complications with generating a *flu* knockout mutant that have caused previous attempts to fail.

Adequate antibiotic concentrations in agar plates is important for selection of colonies with both pCasRed and pCRISPR-SacB-gDNA plasmids. Some of the chloramphenicol/ kanamycin antibiotic plates used were observed to have a thin agar thickness, which could lead to a sub-lethal concentration of antibiotics or increased susceptibility to antibiotic degradation during plate storage. This may allow for survival of cells that were not transformed with both plasmids for expression of chloramphenicol and kanamycin resistance genes and would not be able to have any genome editing. This could be a reason that explains the observation that without any use of arabinose, plated tiny colonies were not able to survive transfer on fresh antibiotic media. Ensuring proper antibiotic selection would reduce the number of background unedited colonies.

The design of high efficiency gRNA targets for Cas9 cleavage would reduce escapers. One way to test gDNA quality is to assess if there is a decrease in the number of escaper colonies with transformed pCRISPR-SacB-gDNA compared with empty pCRISPR-SacB (9). CHOPCHOP v2 is an online tool that was used in this study to assess if the gRNA target was suitable for CRISPR/Cas9-directed mutagenesis (12). The designed gDNA had no self-complementarity or predicted off-target effects, suggesting that it was specific to the target locus. However, the gDNA sequence was determined to have an efficiency score of 0.47, ranking 331 out of 537, using the default Xu *et al.* metric (13). This suggests that the site used had low Cas9 cutting efficiency, which may have impacted the likelihood of successful mutagenesis and reduction of background escapers. Redesigning the gDNA for mutagenesis at a high efficiency site may improve the rate of success in generating the *flu* knockout.

In addition to transformation with plasmids for expression of gRNA and Cas9, arabinose induction for lambda Red machinery expression is important to successfully generate a knockout mutant. This allows for repair of the CRISPR/Cas9 double-stranded break by recombination with the dDNA. Cells transformed with both pCasRed and pCRISPR-SacB-gDNA, but not induced with arabinose, formed very small colonies (<1 mm diameter) and could not be continually cultured when transferred to fresh selective plates. There are three steps where arabinose induction can be performed: the culture for preparation of chemically competent cells, the recovery period after transformation, and plating of cells after recovery. We observed that arabinose induction during recovery and plating was sufficient for transformants to be successfully transferred and continually cultured on new plates, in addition to growing to larger colony sizes (up to 4 mm). However, earlier induction of lambda Red genes with arabinose during the preparation of chemically competent cells produced significantly more colony forming units, while retaining the characteristics of large colony size and transferability. This suggests that a longer period of expression of lambda Red recombination machinery prior to transformation and expression of pCRISPR-SacB-gDNA may benefit survival of the cells, and may improve the rate of success when generating mutants. This study showed that arabinose induction of the lambda Red recombination genes within the pCasRed plasmid is necessary for generating the mutant strain.

Improving donor DNA transformation may increase recombination efficiency for generation of knockout mutants. Previous studies have shown that transformation of linear DNA in *E. coli* is inefficient due to exonuclease activity after the DNA enters the cell (18). However, electrocompetent cells and electroporation improves the transformation of linear double-stranded DNA (18). Electroporation of dDNA has previously been performed to successfully generate knockout mutants using the same CRISPR and Cas9 plasmids (11). The use of heat shock transformation of dDNA into chemically competent cells in this study may have prevented successful mutagenesis, due to insufficient dDNA within the cells for recombination. As a result, electroporation may be a better choice for transformation.

Increasing the length of dDNA for transformation may also increase recombination efficiency. The 60 bp dDNA designed for this study is shorter than the 70 bp dDNA design that has been reported to be successful in previous literature (11). This allows for only 30 bp of homologous sequences on either side of the CRISPR-mediated break, rather than the 35 bp of homologous sequences that would have been provided by a 70 bp dDNA. Although it is simpler and more cost-efficient to construct a 60 bp dDNA, shorter homology arms have been reported to decrease the efficiency of homologous recombination by the lambda Red machinery (19). Alternatively, it may be possible to design two oligonucleotides with a central overlapping region, and then PCR extend and amplify to generate a longer dDNA. However, there may be difficulties with oligonucleotide design for PCR suitability, as well as challenges in purifying the dDNA PCR product with sufficient quantity for a single transformation (10 µg). Therefore, it is not recommended to generate the double-stranded dDNA through PCR extension, but instead, to design and construct a 70 bp dDNA from two entirely complementary strands.

Alternative CRISPR/Cas9 systems of genome editing could improve mutagenesis efficiency. *E. coli* can perform recombination even without the lambda phage, using the *rec* genes (14). RecA can improve lambda Red recombination efficiency (19), so increasing expression of recA or even other *rec* genes within a transformed plasmid may increase the chance of generating a knockout mutant. The need for a linear dDNA for homologous recombination can also be eliminated with all the parts needed for CRISPR/Cas9 genome editing in a single plasmid (19). However, for multi-gene editing, using a two-plasmid system may take less time for mutagenesis than a one-plasmid system (20).

In conclusion, we attempted to generate a *flu* knockout mutant by bacterial CRISPR/Cas9 that creates a 30 bp deletion and inserts an in-frame stop codon in the *flu* gene. The efficiency of mutagenesis was lower than expected, suggesting that the protocol used in this study should be optimized. It is recommended that one uses CHOPCHOP v2 for identification of an ideal PAM site, electroporation to improve transformation efficiency, and a 70 bp dDNA to increase lambda Red recombination efficiency.

Future Directions The study describes a method that can be used to generate a *flu* knockout using bacterial CRISPR/Cas9. Although *E. coli* MG1655 has been reported to produce biofilm (21), the MG1655 strain used in this study was found to be unable to produce biofilm (I. Lee, A. Moraes, F. Ma, and D. Chan, submitted for publication). Future studies can be done on biofilm forming bacterial strains and a *flu* knockout from the isogenic strain to examine the role of Ag43 in biofilm.

The CRISPR/Cas9 mutagenesis method as outlined in this study can be used to generate knockout mutants of other genes, as well as in other bacterial strains. However, future work should attempt to improve the protocol to increase the mutagenesis efficiency. Using CHOPCHOP v2 to identify a favourable target site for deletion prior to designing gDNA, dDNA, and primers may improve Cas9 cutting efficiency and possibly increase likelihood of success. Constructing a 70 bp dDNA and using electroporation for dDNA transformation are also ways to improve homologous recombination efficiency. Developing an improved CRISPR/Cas9 system could also be valuable, such as by removing the need for linear dDNA or by including *E. coli rec* genes that increase recombination efficiency of the lambda Red machinery.

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