Journal of Experimental Microbiology and Immunology (JEMI) Vol. 20: 47 – 54

Applying the λ-Red Recombinase System to Generate an *rcsF* Deletion in *Escherichia coli* DH300

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The outermembrane sensor protein, RcsF, is a component of the Rcs phosphorelay pathway of Escherichia coli, which regulates capsule and biofilm formation. RcsF is shown to be necessary to sense peptidoglycan disruption in the event of exposure to subinhibitory concentrations of antibiotics. However, the role of RcsF in sensing other forms of environmental stress is unclear. In this study, we apply the λ-Red recombinase system to knockout rcsF in E. coli DH300, a strain that contains rprA, a gene transcribed downstream of the Rcs pathway, fused with a lacZ reporter. We have transformed the λ-Red recombinase-expressing pKD46 into E. coli DH300 using the Transformation and Storage Solution protocol, and PCR was performed to amplify a chloramphenicol resistance cassette that can be used to knock out rcsF through homologous recombination. The expression of rprA after induction with subinhibitory concentration of cefsulodin was measured using the β-galactosidase assay. Our results confirmed that rprA::lacZ fusion in E. coli DH300 is functional. A 1.55 times increase in rprA expression in the induced sample compared to the uninduced control was observed. This study provides preliminary work for future research investigating the role of RcsF.

Capsule formation and biofilm production in *Escherichia coli* confer nonspecific antibiotic resistance (1). One of the pathways responsible for capsule formation is the Rcs pathway, a phosphorelay system that upregulates the *cps* gene cluster for colanic acid production, the primary component of capsules in *E. coli*, as well as the *rprA* gene cluster for biofilm formation (2, 3). The Rcs pathway in *E. coli* responds to envelope stress such as osmotic shock and subinhibitory levels of antibiotics (4). RcsF is a lipoprotein at the surface of the outer membrane, and it senses cell surface perturbation (4). RcsF is upstream of the classical RcsCDB phosphorelay pathway, activates the pathway, and has been proposed to sense a variety of environmental stress factors (1, 5).

Although Laubacher and Ades determined that RcsF is necessary in sensing peptidoglycan disruption in the event of exposure to subinhibitory levels of antibiotics, the protein's role in sensing other inducers of the Rcs pathway is still unclear (1). Since osmotic shock (in the form of NaCl) induces capsule formation (2, 6), here we investigate whether *E. coli* uses RcsF to sense osmotic shock in addition to its role in detecting subinhibitory levels of antibiotics. We hypothesize that RcsF is necessary to activate the Rcs phosphorelay pathway in the presence of NaCl osmotic shock. The aim of this study is to create a *rcsF* knockout mutant in the background of *E. coli* DH300, a strain containing an genomic *rprA::lacZ* fusion gene (4). Through the *rprA::lacZ* reporter, the role of RcsF in the activation of the Rcs pathway can be easily measured through the ß-galactosidase assay while exposing the strain to different concentrations of NaCl.

The λ-Red recombinase system applied in this study to knock out *rcsF* in *E. coli* DH300 is a technique developed by Datsenko and Wanner that employs phage λ-Red recombinase under the control of an inducible promoter to replace target genes via homologous recombination (7). Our goal is to inactivate chromosomal genes in *E. coli* through homologous recombination with an antibiotic resistance cassette. In this study, we achieved the preliminary steps to knocking out *rcsF* using the λ-Red recombinase system.

MATERIALS AND METHODS

Strains used in this study. *E. coli* DH300 was obtained from the MICB 447/421 culture collection from the department of Microbiology and Immunology at University of British Columbia, and *E. coli* BW25113 *ΔrscF* were supplied by the Coli Genetic Stock Collection (CGSC). The cells were grown at 37°C with shaking at 180 rpm overnight in Luria-Bertani (LB) broth (pH 7.0, 1% w/v tryptone, 0.5% w/v yeast extract, and 1% NaCl).

Plasmids. *E. coli* BW25113 carrying pKD46 (7), *E. coli* BW25141 carrying pKD3 (7), and E. coli DH5α carrying pCR2.1-TOPO-1-1eta_M13R (positive control for PCR) were supplied by the department of Microbiology and Immunology at the University of British Columbia. pKD46 is a 6329 bp plasmid containing the λ-Red recombinase components (7), and pKD3 is a 2804 bp plasmid containing chloramphenicol acetyl-transferase gene (*cat*) (7). *E. coli* BW25113 carrying pKD46 was grown overnight in LB broth containing 100 μg/ml ampicillin at 30°C at 196 rpm because pKD46 is temperature sensitive (7). *E. coli* BW25141 carrying pKD3 was grown overnight in LB broth containing 25 μg/ml chloramphenicol at 37°C at 180 rpm.

Plasmid isolation. The plasmids pKD46 and pKD3 were isolated from *E. coli* BW25113 and *E. coli* BW25141, respectively. The isolation was carried out using the Invitrogen PureLink HQ Mini Plasmid DNA Purification Kit (Cat# K2100-01). DNA purity and concentration were measured using a Thermo Scientific NanoDrop 2000c Spectrophotometer at absorbance of 260 nm and 280 nm.

Minimum inhibitory concentration assay to determine the subinhibitory concentration of cefsulodin. 1 ml of overnight culture of *E. coli* BW25113 and *E. coli* BW25113 *ΔrscF* were transferred into 9 ml of LB broth and grown separately for 2 hours at 37°C with shaking at 180 rpm. Samples were standardized based on OD_{600} readings (8, 9) and diluted to 6.25×10^6 cells/ml. A two-fold serial dilution of the antibiotic cefsulodin was performed in a 96-well plate to obtain wells containing varying concentrations of: 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml, 6.25 μg/ml, 3.12 μg/ml, and 1.06 μg/ml. The plate was incubated at 37°C and shaken at 180 rpm overnight. Minimum inhibitory concentration (MIC) was

qualitatively determined by the well that had the minimum concentration of antibiotic added with no visible growth. Subinhibitory concentration was determined as the wells with bacterial growth at the highest concentration of antibiotic.

Preliminary β-galactosidase assay for detection of LacZ activity in *E. coli* DH300. Two diluted cultures of 0.02 OD_{600} were made by transferring 50 µl of *E. coli* DH300 overnight culture into 9.95 ml of fresh LB broth grown at 37°C with shaking at 180 rpm until 0.45 OD_{600}. Subinhibitory concentration cefsulodin was added to one of the diluted culture to induce *rprA* activity, and the other culture was left untreated. The cultures were placed back into the incubator and checked at 10-minute intervals until they reached 0.5 OD_{600}. As per Miller's protocol for β-galactosidase assay (10), 2 ml of cells from each culture were pelleted at 6000 rpm for 10 min at 4°C. The supernatant was discarded and replaced with 2 ml of chilled Z buffer (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol). The cells were re-suspended, and the OD_{600} reading for each sample was measured and recorded. Next, 0.5 ml of the cell suspension from each sample was transferred into 0.5 ml of Z buffer to dilute the samples to 1 ml. The cells in each sample were permeabilized by adding 100 µl chloroform and 50 µl 0.1% SDS. The samples were then vortexed and incubated for 5 minutes in 28°C water bath. To begin the reaction, 200 µl of *o*-nitrophenyl-β-D-galactoside (ONPG; 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mg/ml o-nitrophenyl-β-D-galactoside) were added to each sample. The samples were mixed by vortexing, and time of ONPG addition to each sample was recorded. The samples were incubated in 28°C water bath until a sufficient yellow colour had developed, upon which 0.5 mL of 1M Na_2CO_3 was added to each sample to stop the reaction. The samples were vortexed and the time of the end of each reaction was recorded. The samples were spun at 16,000 rcf for 5 minutes to remove debris and chloroform. The supernatant for each sample was transferred into a cuvette, and the A_{420} and A_{550} absorbance readings were measured using a Biochrom Ultrospec 3000 spectrometer. The absorbance values were then converted to Miller Units. The Miller Unit is an arbitrary unit used to represent and compare β-galactosidase expression (10). Miller Unit was calculated using the equation below according to the method of Miller (10): Miller Units = 1000 x (OD_{420} - 1.75 x OD_{550}) / (Time of reaction x Volume of culture x OD_{600}).

Transformation of λ-Red recombinase expressing pKD46. The TSS transformation protocol described by Chung et al. was followed (11). *E. coli* DH300 overnight culture was diluted 1/100 times by adding 0.1ml of overnight culture to 9.9 ml of LB broth, and the diluted culture was grown at 37°C at 180 rpm until 0.3-0.4 OD. An aliquot of 2 ml was taken from the diluted culture and equal volume of 2X Transformation and Storage Solution (TSS) was added to produce competent cells. 100 µl of competent cells were added to 500 ng of isolated pKD46 in an Eppendorf tube and the mixture was left at 4°C to transform for 1.5 hours. After the transformation step was completed, 0.9 ml of 1X TSS was added to the cells, making the final volume 1 ml. The cells were recovered at 30°C and shaken at 196 rpm for 6 hours. Then, the transformants were spun down at 16,000 rcf for 90 seconds and 0.9 ml of supernatant was discarded. The transformants were resuspended in the remaining 100 µl of TSS and plated on LB agar plate containing 100 µg/ml ampicillin. The plate was incubated at 30°C overnight.

Gradient PCR amplification of chloramphenicol Acetyl-Transferase (*cat*) region in pKD3. The sequence of forward primer used was 5'-GCCTGACGATAGCAGCCTGGCGTACCG CTGGTGACTTCGCGTGTAGGCTGGAGCTGCTTC-3', and the sequence for reverse primer was 5'-CCCGTTCAAAGCACTGCACCCCAGCCGAAAGCGGAGCC TGATGGGAATTAGCCATGGTCC-3'. The primers were ordered from Integrated DNA Technologies (IDT). All gradient PCR reactions were carried out in volumes of 50 µl, and each reaction contained 2 µl of DNA template at 40-50 ng/µl, 5 µl of 10X polymerase buffer, 5 µl of 50 mM $MgCl_2$, 1 µl of 10 mM dNTPs, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 34.6 µl PCR grade water, and 0.4 µl Platinum® Pfx DNA Polymerase (Invitrogen, Cat#10966018). Gradient PCR thermocycling conditions were 2 minutes of initial denaturation at 94°C followed by 35 cycles of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 50°C-65°C, 30 seconds of extension at 72°C, and a final extension at 72°C for 5 minutes. PCR products were purified using PureLink® PCR Purification Kit (Invitrogen, Cat# K310001), and the DNA purity and concentration were measured by a Thermo Scientific NanoDrop 2000c Spectrophotometer at absorbance of 260 nm and 280 nm. PCR products were run on 1% agarose gel in 1X TBE buffer at 110 V for 60 minutes. Bands were visualized using ethidium bromide.

Chloramphenicol cassette transformation. *E. coli* DH300 transformants carrying pKD46 produced using TSS transformation protocol (11) were grown in LB broth with 100 µg/ml ampicillin at 30°C and shaken at 196 rpm overnight. The overnight culture was diluted 1/100 times by adding 0.1 ml of overnight culture to 9.9 ml of LB broth containing 100 µg/ml ampicillin, and the diluted culture was grown at 30°C at 196 rpm until 0.1 OD. 10 mM of L-arabinose were added to the culture to induce λ-Red recombinase expression from pKD46. The cells were incubated at 30°C and shaken at 196 rpm to allow growth until OD 0.3-0.4. At this point 2 ml of the culture was taken out and mixed with 2 ml of 2X TSS and 100 µg/ml ampicillin. After the competent cell preparation steps were completed, 100 µl of competent cells were added to an Eppendorf tube and mixed with 500 ng of linear PCR products, containing *cat* region of pKD3 with flanking regions targeting *rcsF* in *E. coli* DH300. The cells were left to transform at 4°C for 10 minutes (12, 13). The tubes were then transferred to room temperature and incubated for 10 minutes. Lastly, the cells were placed at 4°C to incubate for another 10 minutes (12, 13). After the transformation steps had been completed, 0.9 ml of SOC with 100 µg/ml ampicillin was added to the transformed cells. The cells were recovered at 30°C with shaking at 196 rpm for 90 minutes. The culture containing the possible transformants was spun down at 16,000 rcf for 90 seconds, and 0.9 ml of the supernatant was discarded. The cells were resuspended in the remaining 100 µl of SOC and plated on LB agar plates containing 25 µg/ml chloramphenicol. The plates were grown at 37°C incubator since pKD46 was no longer needed.

Colony PCR amplification of *rcsF* region to verify *rcsF* deletion. The sequence of forward primer, delRcsF, used was 5'-GCTCCTGATTCAATATTGACG-3', and the sequence for reverse primer used was 5'-CTATTTGCTCGAACTGGAAAC-3'. The primers were obtained from the CGSC (14). For the positive control, the primers, AJAE, were (forward) 5'-CATCCATGGCTATGGACGTT-3' and (reverse) 5'-CTTGCCGGCCGCATTATTAC-3'. Colony PCR reactions were carried out in volumes of 50 µl. Colonies of interest were gently touched by pipette tip and mixed with 5 µl of 10X polymerase buffer, 5 µl of 50 mM $MgCl_2$, 1 µl of 10 mM dNTPs, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 36.6 µl PCR grade water, and 0.4 µl Platinum® Pfx DNA Polymerase (Invitrogen, Cat#10966018). PCR conditions were 2 minutes of initial denaturation at 94°C followed by 35 cycles of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 52°C, 30 seconds of extension at 72°C, and a final extension at 72°C for 5 minutes. PCR products were run on 1% agarose gel in 1X TBE buffer at 110 V for 60 minutes. Bands were visualized using ethidium bromide.

DNA sequencing of PCR amplification of *rcsF* region from chloramphenicol resistant transformant. PCR products consisting of *rcsF* region in chloramphenicol resistant

transformants were purified using PureLink® PCR Purification Kit (Invitrogen, Cat# K310001). 10 µl of PCR products at15 ng/µl along with 5 µl of delRcsF primers at 5 pmol/µl were prepared as requested by NAPS (Nucleic Acid Protein Service) unit for Sanger sequencing. The sample and primers were delivered to NAPS on the UBC campus.

RESULTS

TABLE 1 Minimum inhibitory concentration (MIC) and subinhibitory concentration of cefsulodin for treatment of *E. coli* BW25113 wild type and mutant strain at 37°C overnight.

Strain	MIC (µg/ml)	Subinhibitory Concentration (µg/ml)
BW25113	12.5	6.25
BW25113 *ΔrcsF*	12.5	6.25

The subinhibitory concentrations of cefsulodin. We wanted to investigate whether the *rprA::lacZ* fusion was functional in *E.coli* DH300 by inducing *rprA* expression using subinhibitory concentration of antibiotic. In order to determine the subinhibitory antibiotic concentration needed to induce *lacZ* reporter gene, we performed a cefsulodin MIC assay on the Δ*rcsF* mutant in triplicates. Overnight cultures of *E. coli* BW25113 wild type and mutant Δ*rcsF* were standardized to $6.25x10^6$ cells/ml. Cefsulodin underwent two-fold serial dilutions in a 96-well plate to obtain wells containing varying concentrations of antibiotic. A positive control for growth inhibition contained 100 µg/ml cefsulodin, whereas a negative control contained no antibiotic. The positive control showed no evidence of growth, and the negative control showed growth. These controls confirmed that the MIC assay had no contaminants, and that the results are valid. Both the *E. coli* BW25113 wild type and Δ*rcsF* mutant share the same minimum inhibitory concentration, 12.5 µg/ml, for cefsulodin (Table 1). Therefore, the subinhibitory concentrations for both the wild type and mutant were determined to be 6.25 µg/ml (Table 1).

***E. coli* DH300 *rprA* gene expression is increased following subinhibitory cefsulodin pretreatment.** To quantify the magnitude of *rprA* expression, we measured the LacZ activity by conducting the β-galactosidase assay. We used the subinhibitory concentration of cefsulodin, 6.25 µg/ml (Table 1), to induce *rprA* expression in *E. coli* DH300. Overnight *E. coli* DH300 culture was diluted and grown at 37°C in two separate cultures to OD_{600} 0.45, at which point only one culture was induced with cefsulodin. The β-galactosidase assay was conducted in duplicate and the absorbance at 420 nm and 550 nm were measured and used to calculate Miller Units (10). The *E. coli* DH300 culture that was left untreated with cefsulodin acted as the negative control to provide a reference to the baseline *rprA* expression. The results from the preliminary β-galactosidase assay showed that the induced sample had 36.09 Miller Units whereas the baseline expression for *rprA* was calculated to be 23.29 Miller Units (Table 2).

TABLE 2 *rprA* gene expression of *E. coli* DH300 wild type with and without induction with a subinhibitory concentration of cefsulodin

Strain	Cefsulodin (+/-)	β-Gal activity (Miller unit)
DH300 WT	+	36.09
DH300 WT	-	23.29

Therefore, according to the β-galactosidase assay, the induced sample was 1.55 times higher compared to the uninduced control (Table 2). The results confirmed that the *rprA::lacZ* fusion was functioning, and the level of *rprA* expression in *E.coli* DH300 was increased through induction using a subinhibitory antibiotic concentration.

pKD46 containing ampicillin resistance gene was transformed into *E. coli* DH300. After confirming the *lacZ* reporter is functional in *E. coli* DH300, we proceeded to use the λ-Red recombinase system to knock out *rcsF* in *E. coli* DH300. In order for the antibiotic cassette to undergo homologous recombination and replace *rcsF*, the λ-Red recombinase expressing pKD46 needed to be transformed into *E. coli* DH300 using the TSS protocol. An overnight culture of *E. coli* DH300 was made competent following the TSS method (11), and the competent cells underwent transformation and recovery phases. Lastly, the transformants were plated on LB plates with 100 µg/ml ampicillin and incubated at 30°C. As a positive control, pUC19 which also confers ampicillin resistance, was transformed into *E. coli* DH300. As a negative control for growth on ampicillin plates, untransformed *E. coli* DH300 were streaked onto a LB plate containing 100 µg/ml ampicillin and incubated at 37°C. After overnight incubation, we obtained isolated colonies on both experimental and positive control plates. The negative control for growth did not show any evidence of growth which indicated that untransformed *E. coli* DH300 were sensitive to ampicillin. The positive control confirmed that the *E. coli* DH300 cells were competent. Since *E. coli* DH300 does not have ampicillin resistance, the growth on ampicillin plates indicated we had obtained ampicillin resistant *E. coli* DH300 containing pKD46, which could express recombinase upon induction by L-arabinose (7).

Confirmation of PCR amplification of the Chloramphenicol Acetyl-Transferase (*cat*) region in pKD3. PCR was performed on pKD3 with primers that flanked the *cat* gene and were complementary to regions of the DH300 genome flanking the *rcsF* coding sequence. The resulting amplicon, when transformed into DH300 expressing the λ-Red recombinase, should undergo homologous recombination with the DH300 genome and delete *rcsF* (Figure 1). Gradient PCR was performed to experimentally determine the optimum annealing temperature for the primers, and gel electrophoresis was performed. The positive control for PCR was pCR2.1-TOPO-1-1eta-M13R carrying Potato Proteinase Inhibitor II gene, *PI2*, which is 462 bp (15). Single bands of different strength appeared around the 1 kb region in lanes 2 to 7 (Figure 2), which corresponds to the expected bands for *cat*,

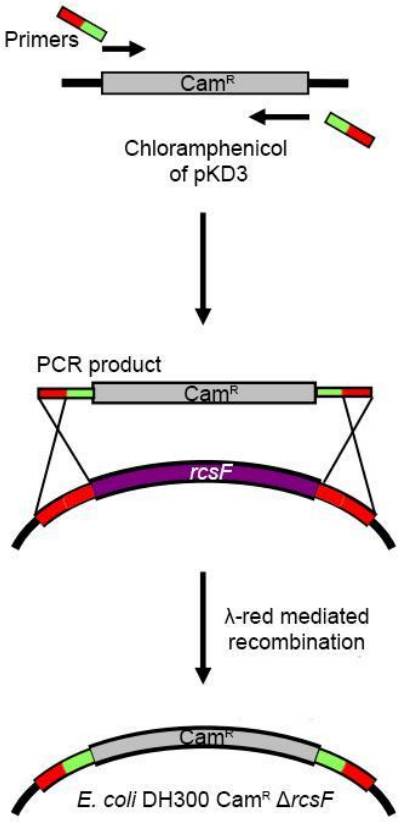

FIG 1 Illustration of λ-Red recombinase system to knockout *rcsF* using the PCR product containing the chloramphenicol resistance gene (Adapted from Beamish *et al.* (19)).

since the size of *cat* is 1100 bp (7). The optimal annealing temperature of our primers was 55°C, at which most PCR products were amplified. The band at around 500 bp in lane 8 matched the expected size of the positive control, 462 bp. The positive control indicated that the PCR conditions were adequate, and the results confirmed that we had amplified the *cat* cassette from pKD3.

Chloramphenicol-resistant colony from the transformation of *cat* into DH300/pKD46 was not Δ*rcsF*. Finally, the last step to knocking out *rcsF* in *E. coli* DH300 was to transform linear chloramphenicol resistance cassettes that we obtained from PCR amplification into previously transformed *E. coli* DH300 containing pKD46 using the TSS protocol (11). Our goal was to achieve homologous recombination between the chloramphenicol resistance cassette and *rcsF* (Figure 1). 10 mM of L-arabinose were added to *E. coli* DH300/pKD46 transformants to induce λ-Red recombinase components in pKD46. The cells were then made competent, and the linear PCR products were added to competent cells. After the transformation and recovery phases, the cells were plated on LB plates with 15 μg/ml chloramphenicol and incubated at 37°C to cure the cells of pKD46. For the positive control, pKD3 were transformed into *E. coli* DH300/pKD46 and plated on LB plates containing 15 μg/ml chloramphenicol.

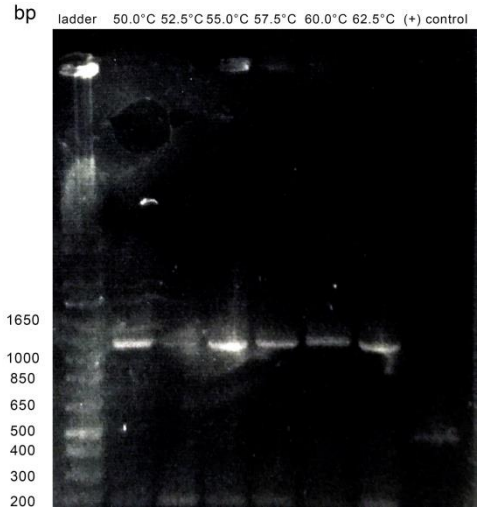

FIG 2 Amplification of *cat* region from pKD3 using gradient PCR and visualized in 1.0% agarose gel, running in 1X TBE buffer at 110V for 1 hr.

Initially, a concentration of 25 μg/ml chloramphenicol was used in the LB plates, however this condition yielded no growth so the chloramphenicol concentration was reduced. The positive control yielded isolated colonies, which indicated that the *E. coli* DH300/pKD46 cells were competent. The transformation was repeated, but this time with the lower chloramphenicol concentration to reduce the antibiotic stress on cells that may be expressing the chloramphenicol resistance gene at a low rate. We also used a negative growth control, in which *E. coli* DH300 were streaked onto LB plates containing 15 μg/ml of chloramphenicol. The negative control showed no evidence of growth, but our experimental plates yielded one isolated colony. We performed a PCR analysis on the possible transformant with primers flanking *rcsF*, and visualized the DNA through gel electrophoresis (Figure 3). We were expecting bands at 435 bp for WT, and 1100 bp for our transformant. However, PCR amplification of both the wild type and transformant resulted in bands at ~500bp. This was in contrast with our control of known *E. coli* BW25113 *ΔrcsF* in lane 4 (Figure 3), which resulted in a band at the expected 1600 bp (16). The PCR products, which were ~500 bp, amplified from the transformant were sent for Sanger sequencing. The sequencing results indicated that the PCR products were 435 bp (Suppl. 1). A nucleotide BLAST was performed and it showed that the PCR products had 100% identity to the *rcsF* sequences (17). These results all suggest that the chloramphenicol-resistant colony obtained from the experimental plate was not *E. coli* DH300 Δ*rcsF* transformant.

DISCUSSION

We hypothesized that the *E. coli* BW25113 *ΔrscF* mutant strain would be more sensitive to antibiotics, as it is missing the first sensor of the Rcs pathway which leads to capsule formation and higher antibiotic resistance (2).

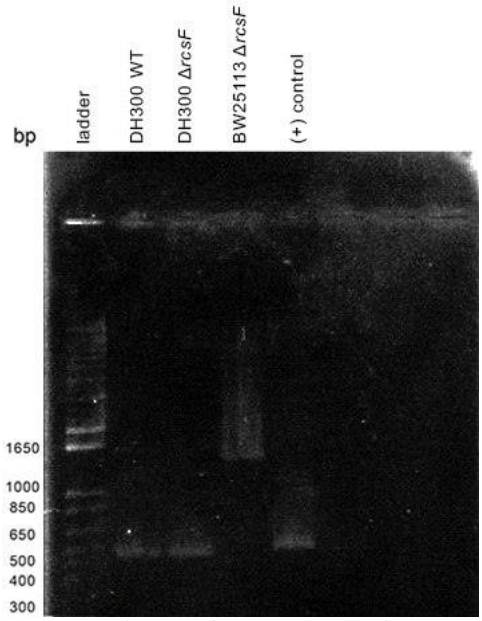

FIG 3 Amplification of the *rcsF* region of possible *E. coli* DH300 *ΔrcsF* using colony PCR visualized in 1.0% agarose gel electrophoresed in 1X TBE buffer at 110V for 1 hr.

The lack of RcsF in the mutant strain did not affect the results of MIC assay, which is unexpected as it seems to suggest that the Rcs pathway was not upregulated enough to show a difference in MIC. This result is most likely due to the assay not being sensitive enough, as the two-fold intervals of increasing cefsulodin concentration for the MIC assay may be too large to show a difference between the wild type and the mutant. Multiple repetitions of the assay should be conducted to ensure for more precise and accurate results.

The MIC assay was done on *E. coli* BW25113, which shares a common ancestor with *E. coli* DH300, *E. coli* W1485 (14). Thus, the MIC results should be considered cautiously. Using the subinhibitory concentration of antibiotic determined by MIC, the β-galactosidase assay results showed that there is a difference in *rprA::lacZ* fusion gene expression due to cefsulodin exposure in *E. Coli* DH300. Since *rprA* is positively regulated by the Rcs pathway, our preliminary β-galactosidase assay confirms Ades and Laubacher's findings that the Rcs pathway is activated in the presence of the antibiotic cefsulodin (1). The β-galactosidase assay also shows that *E. coli* DH300 has a functional *rprA::lacZ* fusion gene and that the strain is suitable for further investigation of the activation of the pathway through the β-galactosidase assay. Another negative control that could have been included was an *E. coli* strain with a *lacZ* reporter that is expressed under a promoter that does not respond to antibiotic induction.

There are many difficulties associated with using chemical transformation (in particular, TSS) to induce linear DNA product uptake into cells. No mutants were obtained from the second transformation even though the experiment was repeated several times with changes to the transformation and recovery times. The difficulty of inducing linear DNA uptake into cells may be due to the approach that was taken for transformation. In our experiment, the TSS protocol was used for both transformations. However, it has been shown several times in the past (7, 18, 19) that electroporation seems to be the conventional transformation method for the λ-Red protocol, and thus may have higher success rate. This may be due to electroporation having a higher transformation efficiency compared to chemical transformation (13). Greater uptake of these linear products may be needed in order for recombination to occur. Methods such as electroporation and Inoue (preparation of ultra-competent cells) are able to induce high numbers of linear products to enter cells to increase the chance of recombination (13, 19).

There are a few possible reasons for chloramphenicol resistant contamination on the plates. Perhaps the single isolated colony that survived is a pKD3 harbouring bacteria, as the amplified PCR product used for the second transformation still contained trace amounts of pKD3 that served as template DNA. Although unlikely, the cells used for the second transformation may have also spontaneously mutated and became chloramphenicol resistant. In addition, chloramphenicol is a bacteriostatic antibiotic, stopping bacterial growth by inhibiting protein synthesis (20). It is possible that some bacteria were able to grow again after the antibiotic somehow degraded, perhaps due to exposure to light as chloramphenicol is light sensitive (13).

We have confirmed some previous findings from literature and taken the first steps in creating a *rcsF* mutant for *E. coli* DH300. Although the MIC is the same for both a wild type *E. coli* strain and its corresponding mutant lacking *rcsF*, the higher β-galactosidase activity for *E. coli* induced with cefsulodin shows that the antibiotic is indeed capable of inducing the Rcs pathway. pKD46 was successfully transformed into *E. coli* DH300, and a linear PCR product containing the chloramphenicol resistance gene, *cat*, flanked by *rcsF* regions has been purified. We were unsuccessful with the last steps of the knockout process, which is obtaining and verifying a transformant that now has the *rcsF* replaced with the chloramphenicol resistance cassette through a second transformation and verification through colony PCR.

FUTURE DIRECTIONS

The main difficulty of this study was obtaining a mutant that contains the chloramphenicol resistance cassette in the place of *rcsF*. Although it is uncertain whether the problem is getting the linear PCR product to enter the cells, or the actual homologous recombination between the PCR product and *rcsF*, we propose several changes to increase the

probability of obtaining transformants. Other transformation methods, such as electroporation and the Inoue method of preparing ultra-competent cells, can be performed for the transformation of the linear PCR product (21). Furthermore, a higher concentration of linear PCR product can be added. To avoid contamination with chloramphenicol resistant colonies, cells could be grown on plates with a higher concentration of the selective antibiotic.

Following validation of the *rcsF* deletion with PCR, the focus of future experimental work would be determining the role of RcsF in detecting and responding to antibiotic and environmental stress. A β-galactosidase assay on *E. coli* DH300 Δ*rcsF* and wild type should first be performed, in respect to the antibiotic cefsulodin. The expected results, confirming what is already shown in literature, would be the wild type *E. coli* DH300 showing higher levels of β-galactosidase activity than the mutant (1). Another β-galactosidase assay on *E. coli* DH300 Δ*rcsF* and wild type, now in respect to NaCl osmotic shock, can then be performed. The expected results which would support our hypothesis are that the wild type *E. coli* DH300 shows higher levels of β-galactosidase activity than the mutant. This would demonstrate that the Δ*rcsF* is unable to sense osmotic stress. Different concentrations of NaCl and other salts can also be used to further study the effects of osmotic shock, and determine if *rcsF* is specific for certain molecules at certain concentrations

It is important to note that although we chose to delete *rcsF* in a strain that contains a *rprA::lacZ* reporter in its genome, an alternative way of obtaining a *rcsF* mutant with a *rprA::lacZ* reporter is to order a *rcsF* knockout strain from the Keio collection (i.e. *E. coli* BW25113 Δ*rcsF*) and transform a plasmid containing the reporter into the strain. The Keio mutant should be verified with PCR to confirm the presence of a 1.6 kb band (length of the kanamycin cassette) instead of a 435 bp band using the *rcsF* primers, before continuing on with the abovementioned experiments.

ACKNOWLEDGEMENTS

This study was financially supported by the Department of Microbiology and Immunology at the University of British Columbia. We would also like to thank the Yale Coli Genetic Stock Collection for providing us with *E. Coli* BW25113 *ΔrscF*. The authors would also like to thank Dr. David Oliver, Andrew Santos, Céline Michiels, and Dr. Danielle Krebs for their guidance and support.

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SUPPLEMENTAL MATERIAL

>DNA-delRcsF-F 448 18 411 0.05
NNNNNNNANNAANNCTATGCGTGCTTTACCGATCTGTTTAGTAGCACTCATGCTAAGCGGCTGTTCCATGTTAAGCA
GATCCCCTGTCGAACCCGTTCAAAGCACTGCACCCCAGCCGAAAGCGGAGCCTGCAAAACCGAAAGCGCCGCGCGCC
ACGCCGGTCCGAATTTATACCAATGCAGAAGAATTAGTCGGCAAACCGTTCCGCGATCTCGGTGAAGTCAGTGGCGA
CTCTTGCCAGGCCTCTAATCAGGACTCTCCGCCGAGCATTCCAACCGCACGTAAGCGGATGCAAATCAACGCCTCTA
AAATGAAAGCCAATGCTGTATTACTGCATAGCTGCGAAGTCACCAGCGGTACGCCAGGCTGCTATCGTCAGGCTGTA
TGTATCGGTTCTGCGCTTAACATTACGGCGAAATGAGCAGTTTCCAGTTCNNNNCNAAATAGA

>DNA-delRcsF-R 445 29 411 0.05
NNNNNNNNNGTTAGCGCAGACCGATACATACAGCCTGACGATAGCAGCCTGGCGTACCGCTGGTGACTTCGCAGCTA
TGCAGTAATACAGCATTGGCTTTCATTTTAGAGGCGTTGATTTGCATCCGCTTACGTGCGGTTGGAATGCTCGGCGG

AGAGTCCTGATTAGAGGCCTGGCAAGAGTCGCCACTGACTTCACCGAGATCGCGGAACGGTTTGCCGACTAATTCTT
CTGCATTGGTATAAATTCGGACCGGCGTGGCGCGCGGCGCTTTCGGTTTTGCAGGCTCCGCTTTCGGCTGGGGTGCA
GTGCTTTGAACGGGTTCGACAGGGGATCTGCTTAACATGGAACAGCCGCTTAGCATGAGTGCTACTAAACAGATCGG
TAAAGCACGCATAGTATTTCCTCAATGTATGATCAAAACGTCAATANNNNNCCAGGANCN

FIG S1 Sequencing results for *rcsF* using the forward and reverse primers, delRcsF.