



Construction of *luxCDABE* reporter plasmids to investigate regulation of the *acrAB* and *acrEF* operons by the AcrS repressor in *Escherichia coli* BW25113

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SUMMARY The *acrAB* and *acrEF* operons of *Escherichia coli* encode multidrug efflux pumps capable of efficiently exporting a wide variety of antibiotics. The repression of *acrAB* and *acrEF* by regulator, AcrS, has not yet been completely elucidated. In order to investigate the role of AcrS in the regulation of the *acrAB* and *acrEF* operons, reporter plasmids containing the promoter sequences of these operons were constructed. Promoter regions of *acrAB* and *acrEF* were first amplified and stored in the pCR™2.1-TOPO® vector. Sequencing confirmed that the complete promoter regions of both *acrAB* and *acrEF* were successfully cloned. In order to assess regulation of this system in future experiments, the PCR-amplified promoters were cloned into pCS26. The pCS26 vector contains a promoterless reporter *luxCDABE* operon. Here, we report that we successfully cloned the *acrAB* and *acrEF* operon promoter upstream of the *luxCDABE* operon in pCS26 using Gibson assembly. These constructs can be used in future experiments monitoring change in *acrAB* and *acrEF* gene expression in response to AcrS.

INTRODUCTION

The *acrAB* and *acrEF* operons of *Escherichia coli* encode multidrug efflux pumps capable of efficiently exporting a wide variety of antibiotics (1). Multidrug efflux pumps are a major driver of antibiotic resistance and a thorough understanding of the regulation of efflux pumps is essential in the development of interventions to overcome resistance. AcrB and AcrF are inner membrane transporters which energetically pump a wide variety of compounds to the extracellular space, including tetracyclines, quinolones, and tigecycline (2). Both AcrAB and AcrEF are TolC-dependent; AcrA and AcrE are periplasmic proteins which connect AcrB and AcrF, respectively, to TolC: an outer membrane porin (3). The *acrAB* operon is negatively regulated by AcrR, a protein which is divergently transcribed upstream of *acrAB* (4). More recently discovered is the analogous regulatory protein, AcrS, upstream of the *acrEF* operon. AcrS has been shown to repress transcription of both the *acrAB* and *acrEF* operons (5, 6).

Both MIC assays and RT-qPCR have shown that the deletion of *acrS* increases resistance to kanamycin and results in elevated *acrE* expression (6, 7). This indicates that drug tolerance is mediated by AcrS, which represses the *acrEF* operon. In additional experiments, the pMAPS vector, a pBAD24 derivative containing a L-arabinose-inducible *acrS* gene, was constructed and used to induce AcrS overexpression (8). Kanamycin MIC assays using Δ *acrS* Δ *kan* (cells lacking a kanamycin resistance gene) and Δ *acrE* Δ *kan* mutants transformed with the pMAPS vector were then performed. The Δ *acrE* Δ *kan* + pMAPS transformants showed the greatest decrease in MIC (8). In comparison, the Δ *acrS* Δ *kan* + pBAD24 control, lacking both a genomic and plasmid copy of *acrS*, showed a substantial increase in MIC. It was proposed that AcrS acts as a primary repressor of the *acrEF* operon. This is supported by RT-qPCR of *acrE* and *acrA* mRNA in Δ *acrS* Δ *kan* mutants which showed increased *acrE*

Submitted: 13 May 2018

Accepted: 15 Aug 2018

Published Online: 24 August 2018

Citation: O'Neill Z, Bulka O, Marziali M, Nah R. 2018. Construction of *luxCDABE* reporter plasmids to investigate regulation of the *acrAB* and *acrEF* operons by the AcrS repressor in *Escherichia coli* BW25113. JEMI+ 4:1-9

Editor: Julia Huggins, University of British Columbia

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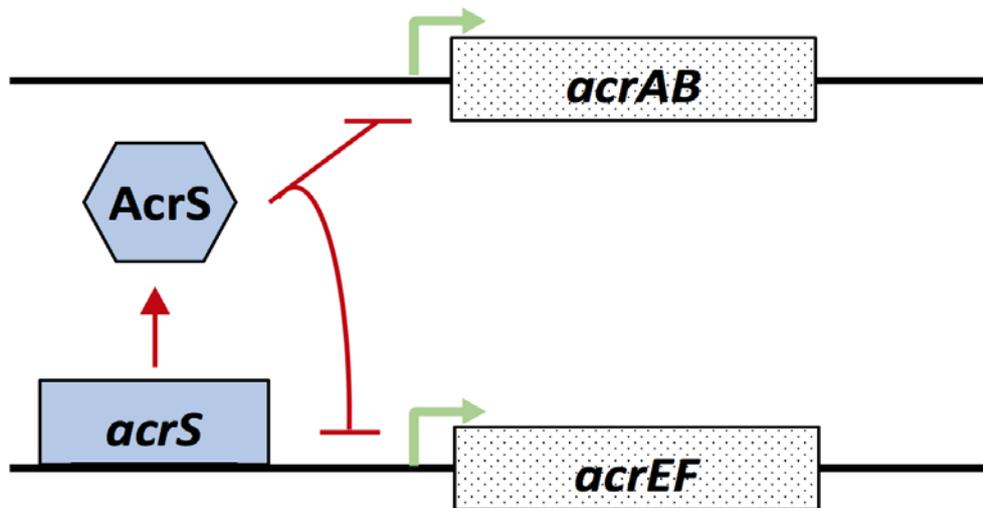


FIG. 1 Proposed model of *acrAB* and *acrEF* transcriptional regulation by repressor AcrS.

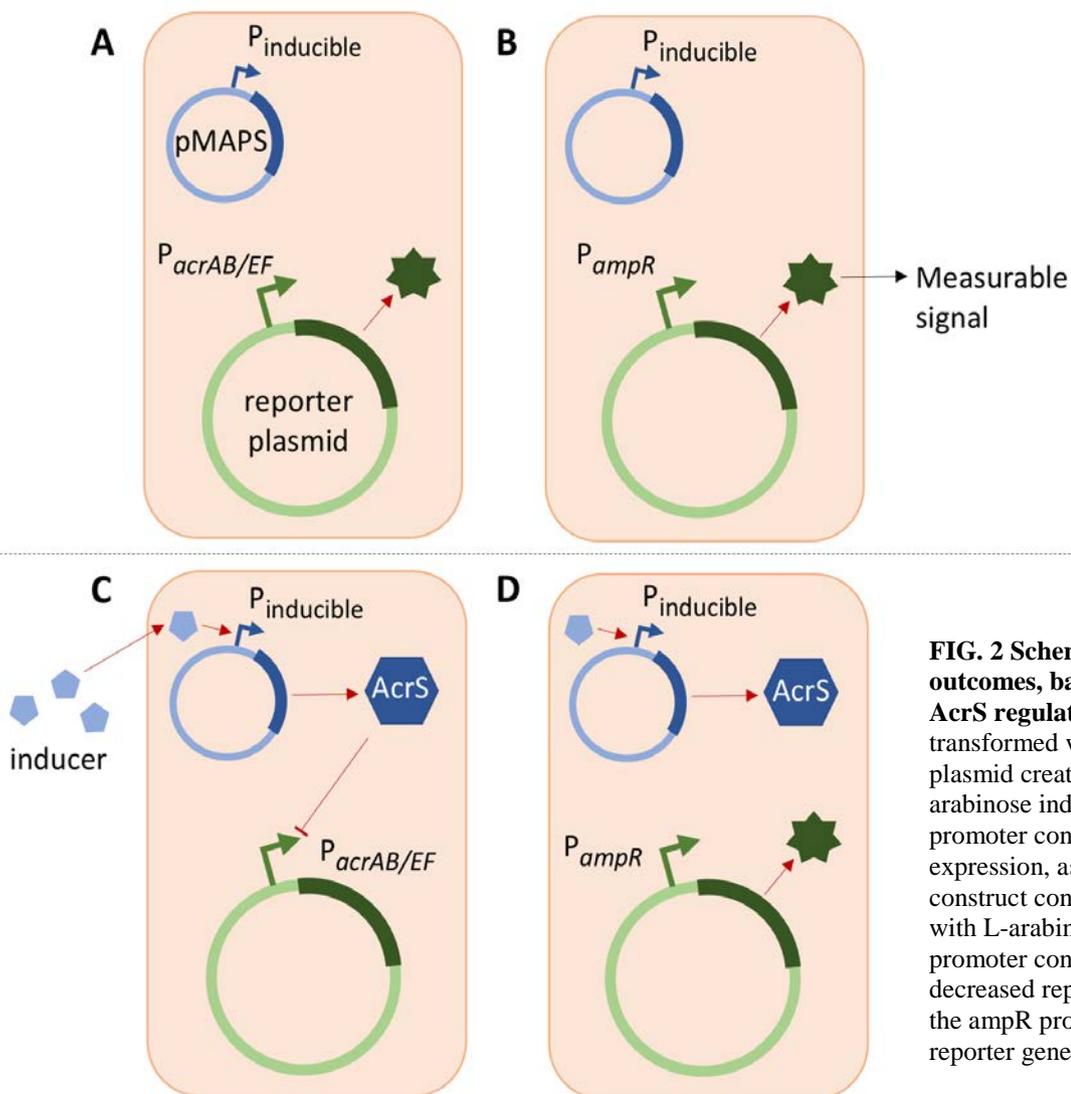


FIG. 2 Schematic of expected project outcomes, based on the proposed model of AcrS regulation. Cells are first co-transformed with pMAPS and the reporter plasmid created in the present study. Before L-arabinose induction, (A) the *acrAB* and *acrEF* promoter constructs should show reporter gene expression, as should the (B) *ampR* promoter construct control. After pMAPS induction with L-arabinose, (C) the *acrAB* and *acrEF* promoter constructs should show no or decreased reporter gene expression, but (D) the *ampR* promoter construct should still show reporter gene expression.

expression compared to *acrE* levels detected in BW25113 (wild type). No difference was observed in *acrA* expression between either the $\Delta\text{acrS}\Delta\text{kan}$ or the $\Delta\text{acrE}\Delta\text{kan}$ mutants and the wild type BW25113 (6).

However, Hirakawa *et al.* report that AcrS represses the *acrAB* operon, but not the *acrEF* operon in *E. coli* (5). AcrS was overexpressed in an ΔacrAB mutant, which did not affect drug susceptibility. These findings were supported through analysis of mRNA transcript levels, which showed that the addition of an *acrS* expression plasmid resulted in a significant decrease in *acrA* transcripts, with little impact on *acrE* transcriptional levels (8).

These variations in findings and proposed models can be explained by a mechanism in which AcrS is acting as a repressor of both the *acrEF* and *acrAB* operons (Fig. 1). Given the contradictory findings of previous work, the role of AcrS as a potential regulator of both the *acrAB* and *acrEF* operons warrants further investigation. The primary objective of this project was to construct reporter vectors that can elucidate whether AcrS represses expression of the *acrEF* operon, the *acrAB* operon, or both (Fig. 2, Fig. 3). The *ampR* gene promoter was chosen to serve as an AcrS-independent control in downstream reporter assays, and thus was also targeted for vector construction.

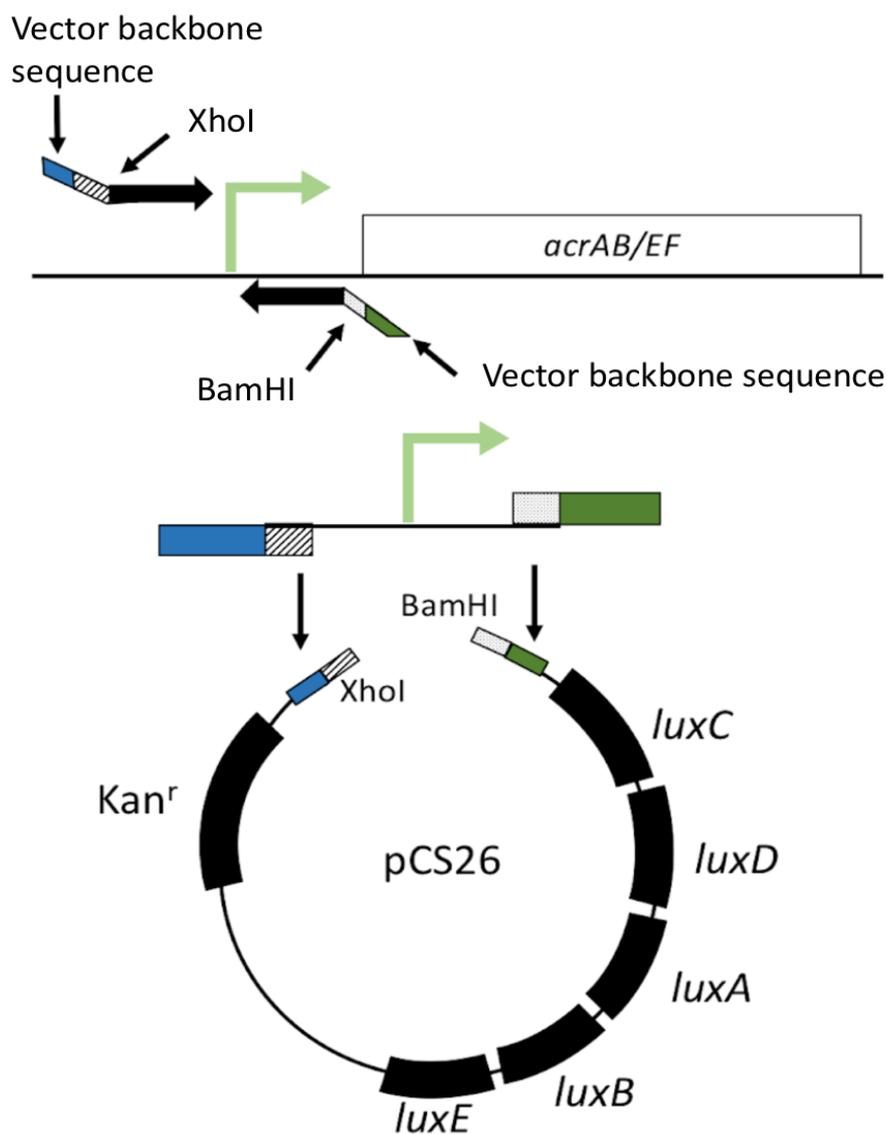


FIG. 3 Cloning strategy. 40bp of vector backbone sequence, including restriction endonuclease sites, are added to each promoter of interest. These are inserted into a digested pCS26 vector, using Gibson assembly.

METHODS AND MATERIALS

Bacterial strains, plasmids and growth conditions. JW3232-1 is a Δ *acrS* single gene knockout strain of *E. coli* K-12 from the Keio strain collection. Chemically competent DH5 α were also used for transformation (LifeTech, Carlsbad, CA). Cells were grown with agitation in lysogeny broth (LB) liquid media at 37°C. LB was supplemented with the appropriate antibiotic for the transformed vector. The following antibiotic concentrations were used: ampicillin at 100 μ g/mL, chloramphenicol at 40 μ g/mL, and kanamycin at 50 μ g/mL.

The pCS26 vector is a 9.3 kb pZS derivative (Addgene plasmid #47640) (10). It is a low-copy-number plasmid containing strong transcriptional terminators and unique promoter cloning sites (XhoI and BamHI) for promoter insertion upstream of a promoterless *luxCDABE* (luciferase) operon from *Photorhabdus luminescens* (11). The pUC19 plasmid is a 2.7 kb high copy number empty backbone cloning vector and was used as a positive control during transformation. As pUC19 contains the *ampR* gene, it was also used as a template for the amplification of the *ampR* promoter region (Addgene plasmid #50005).

Plasmid preparation of pUC19 and pCS26 from carrier strains. Each plasmid host strain was grown in 5 mL of LB broth containing the appropriate selective antibiotic at 37°C overnight. The entire overnight culture was used for plasmid isolation using the PureLink™ Quick Plasmid Miniprep Kit, following the “Purification Procedure using Centrifugation” protocol (Thermo Fisher Scientific, Waltham, MA). All centrifugation steps were performed at room temperature and 15 000 rcf. Deionized water was used for elution in lieu of Tris-EDTA buffer, as EDTA was incompatible with downstream applications. The concentration and purity of isolated plasmid samples were then analyzed using a NanoDrop™ (Thermo Fisher Scientific, Waltham, MA).

Identification of the *acrAB*, *acrEF*, and *ampR* promoter sequences for amplification. The *acrAB* and *acrEF* promoter sequences were previously identified by Hirakawa *et al.* (5). The *acrAB* promoter amplicon includes 317 bp of the region upstream of the *acrAB* +1 site and 82 bp of the region downstream of the transcription start site (5) (Fig. 4). The *acrEF* promoter amplicon includes 318 bp of the region upstream of the *acrEF* +1 site and 81 bp of the region downstream of the transcription start site (Fig. 4). The *acrAB* promoter amplicon contains a 24 bp palindromic region that is directly bound by AcrS (TACATACATT-TATG-AATGTATGTA) (5). The *acrEF* operon also contains a 24 bp region directly bound by AcrS (TCCTTACATCGACGAATGATAATT) (12). The binding locations of the primers were aligned to the *E. coli* BW25113 genome in BLAST. Promoter prediction was accomplished using BPROM (<http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>). The *ampR* promoter sequence was identified by locating the start codon of the *ampR* gene in pUC19. The 200 nucleotides upstream of the start codon contains the promoter (13). Primers were designed to the region 305 bp upstream of the *ampR* transcription start site, and 97 bp of the *ampR* transcript to ensure that the entire promoter would be amplified (Table 1).

TABLE 1 Primer sequences for the amplification of promoters for insertion into pCR™2.1-TOPO® vector

Name	Annealing temp (°C)	Sequence
acrA_Fwd	80	CGCAAGCTTatgtaaacctcgagtgtccga
acrA_Rev	66	CGCGCGCCGCagagtggatcgccagggaa
acrE_Fwd	67	CGCGCGCCGCgattaattattcaggaat
acrE_Rev	59	CGCAAGCTTtactattcctcaaaaaaccaa
ampR_Fwd	80	CGCAAGCTTctcttcctttttcaatattattgaagc
ampR_Rev	69	CGCGCGCCGCtaagccagccccg

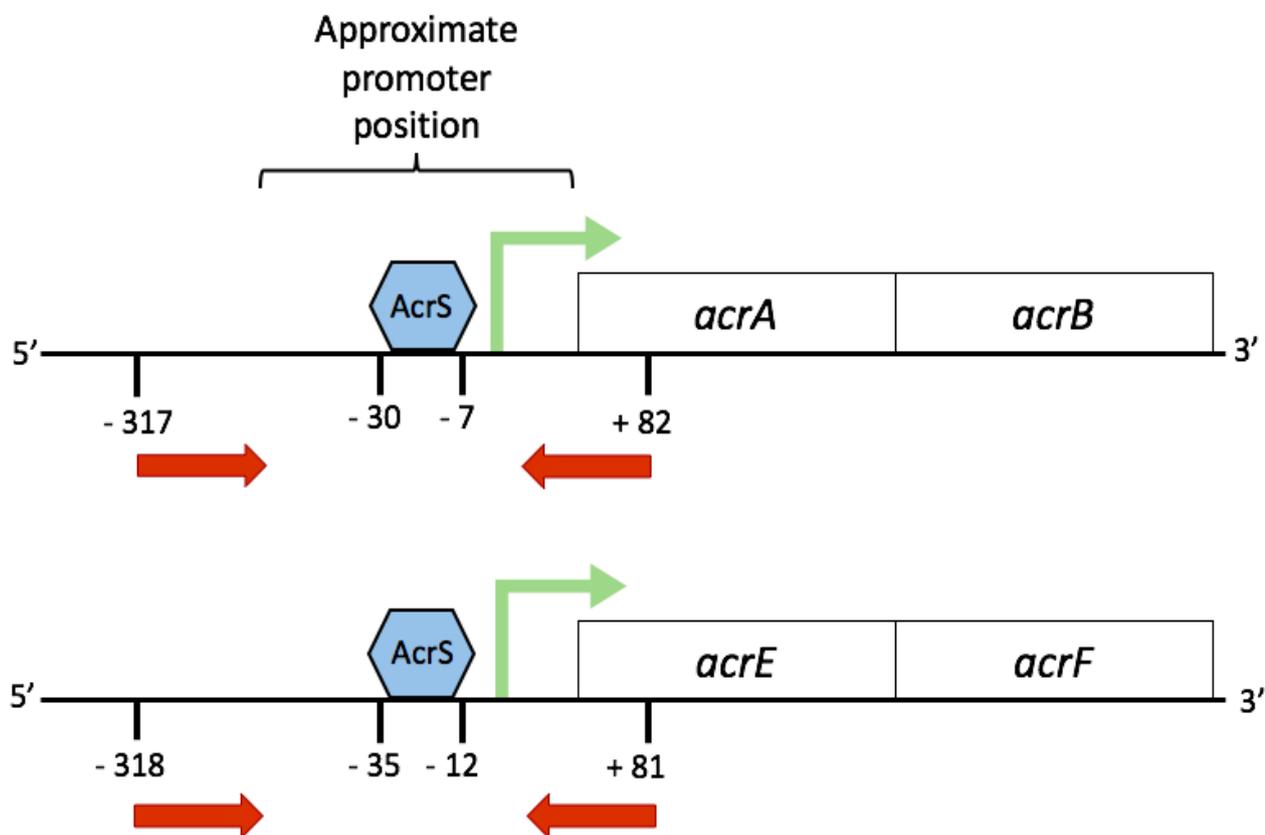


FIG. 4 Characterization of the *acrAB* and *acrEF* promoter regions. Figure based on previous work investigating AcrS regulation of the *acrAB* and *acrEF* operons by Hirakawa *et al.* and this study (5). Primers used for PCR amplification are indicated with red arrows. The relative position of the AcrS binding site (operator) and the predicted position of the overlapping promoter region are indicated.

Amplification of *acrAB*, *acrEF* and *ampR* promoters for cloning into the TOPO® TA vector.

A PCR was run using Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA) to add 3' adenine-overhangs for subsequent TOPO® TA cloning. The *acrAB* and *acrEF* promoters were amplified from BW25113 *E. coli* genomic DNA, using the primers listed in Table 1. The amplification of the *ampR* promoter used pUC19 plasmid DNA as a template. As a PCR positive control, pUC19 was amplified with pUC19 universal primers (pUC19-193F: 5'gtgaataaccgcacagatgc, pUC19-355R: 5'ggcgttaccacaacttaatcg). The PCR reaction components were added according to the Platinum *Pfx* DNA Polymerase manual with the addition of 1% dimethyl sulfoxide (DMSO) (Invitrogen, Carlsbad, CA). To accommodate the high annealing temperature of the *acrAB* and *ampR* primers, the thermocycler was programmed to run a two-step reaction with no annealing step (2 mins at 94°C, 35 cycles of 2 mins denaturation at 94°C, 30 s extension at 68°C, and a final 5 min extension at 68°C). To amplify the *acrEF* promoter, the thermocycler was programmed to run a touchdown PCR (2 mins at 94°C, 15 cycles of 15 s denaturation at 94°C, 30 s annealing initially at 70°C but decreasing incrementally by 1°C each cycle, 30 s extension at 68°C, followed by 19 cycles of 15 s denaturation at 94°C, 30 s annealing at 55°C, 30 s extension at 68°C, and a final 5 min extension at 68°C).

Agarose gel electrophoresis. All PCR products were electrophoresed on 1.5% (w/v) agarose gels at 120V. Gels were made with SYBR® Safe DNA Gel Stain in 0.5x TBE buffer (Thermo Fisher Scientific, Waltham, MA), to allow for visualization of the gel using the Alpha Innotech® MultiImage light cabinet (Alpha Innotech, San Leandro, CA).

PCR purification of the amplified *acrAB*, *acrEF* and *ampR* promoters. The PureLink™ PCR Purification kit was used to purify 40 µL of each PCR sample, using Buffer B2 in the first step (Thermo Fisher Scientific, Waltham, MA). The purified product was then analyzed using NanoDrop™ to determine the concentration of the sample for future applications (Thermo Fisher Scientific, Waltham, MA).

Preparation of chemically competent cells and transformation. Chemically competent DH5α and JW3232-1 cells were prepared following the Hancock protocol for the creation of CaCl₂ competent cells (<http://cmdr.ubc.ca/bobh/method/cac12-transformation-of-e-coli/>). The cells were pelleted after growth to an OD₆₀₀ of 0.1-0.2, and were resuspended in cold 0.1M CaCl₂ for 24 hours. The cells were stored at -80°C in 50% glycerol until transformation, which followed the Hancock protocol for CaCl₂ transformation of *E. coli*.

TOPO® TA cloning and transformation. The amplified promoters were cloned into the pCR™2.1-TOPO® vector following the protocol outlined in the TOPO TA Cloning® Kit (Thermo Fisher Scientific, Waltham, MA). The TOPO® cloning reaction was then transformed into One Shot® TOP10 Chemically Competent *E. coli* cells according to the manufacturer's specifications, and incubated overnight at 37°C on LB-Amp plates (Thermo Fisher Scientific, Waltham, MA).

Amplification of the *acrAB*, *acrEF* and *ampR* promoters for cloning into the pCS26 vector. Primer sets were designed for amplification of the *acrAB*, *acrEF* and *ampR* promoters (Table 2). These primers encoded 40 bp of vector backbone sequence for Gibson assembly into pCS26. The PCR reaction components were added according to the Platinum Pfx DNA Polymerase protocol, with addition of 1% DMSO (Invitrogen, Carlsbad, CA). pCR™2.1-TOPO® vectors containing the *acrAB* and *acrEF* promoters were used as template DNA. Amplification of the *ampR* promoter used pUC19 plasmid DNA as a template. As a PCR positive control, pUC19 was amplified with pUC19 universal primers (pUC19-193F: 5'gtgaaataccgcacagatgc, pUC19-355R: 5'ggcgttaccaacttaacg). To accommodate the low initial annealing temperature of the primer sets, the thermocycler was programmed for a reverse touchdown PCR (2 mins at 94°C, and 15 cycles of 15 s denaturation at 94°C, 30 s annealing initially at 55°C and increasing incrementally by 1°C each cycle, 1 min extension at 68°C, followed by 19 cycles of 15 s denaturation at 94°C, 30 s annealing at 75°C, 30 s extension at 68°C, and a final 5 min extension at 68°C).

TABLE 2 Primer sets designed for amplification of promoter regions and preparation for Gibson assembly

Name	Annealing temp (°C)	Sequence
PacrA_F1	78.5	AAATAGGCGTATCACGAGGCCCTTTCGTCTTCACCTCGAGtatgt aaacctcgagtgtcc
PacrA_R1	80.4	CATATTTGCCATCCATTTTGC GGCCGCAACTAGAGGATCCagagt ggatcgccagggaaat
PacrE_F1	75.5	AAATAGGCGTATCACGAGGCCCTTTCGTCTTCACCTCGAGgatta attattcaggaaata
PacrE_R1	76.7	CATATTTGCCATCCATTTTGC GGCCGCAACTAGAGGATCCtacta ttcctcaaaaaacca
PampR_F1	82.8	AAATAGGCGTATCACGAGGCCCTTTCGTCTTCACCTCGAGtaagc cagccccgacacccg
PampR_R1	80	CATATTTGCCATCCATTTTGC GGCCGCAACTAGAGGATCCgaagg caaaatgccgcaaaa

Gibson assembly cloning of promoter regions into the pCS26 vector. Restriction endonuclease digestion of pCS26 was performed using XhoI (Gibco, Carlsbad, CA) and BamHI (Promega, Madison, WI) in NEB3.1 buffer (New England Biolabs, Ipswich, MA) according to the New England Biolabs double digest protocol (<https://international.neb.com/protocols/2014/05/07/double-digest-protocol-with-standard-restriction-enzymes>).

The reaction was incubated at 37°C for 1 hour, then heat-inactivated at 70°C for 20 min. Gibson assembly was then performed following the manufacturer's specifications (New England Biolabs, Ipswich, MA). The reaction was incubated for 15 min at 50°C.

RESULTS

The promoter regions of *acrAB* and *acrEF* were amplified using PCR. The *acrAB*, *acrEF* and *ampR* promoter regions were each amplified and run on a 1.5% agarose gel (Fig. 5). The positive control (pUC19) lane failed to show the expected band pattern. The negative control lane, containing the pUC19 universal primer set and lacking template DNA, resulted in the expected absence of a band. The gel showed bands at approximately 400 bp in the *acrAB*, *acrEF* and *ampR* amplification lanes. These bands suggest that the three promoter regions were successfully amplified.

The promoter sequences were successfully stored through TOPO cloning. In order to confirm the sequences of the amplified promoters and preserve them for future use, *acrAB*, *acrEF* and *ampR* promoter regions were amplified with *Taq* polymerase and cloned into the pCR™2.1-TOPO® vector. Subsequent sequencing using universal M13R primers confirmed that the DNA regions that were amplified and cloned corresponded to the complete promoter regions of *acrAB* and *acrEF* (Fig. S1). Sequencing also revealed amplification of the *ampR* promoter was partially successful, as a portion of the *ampR* promoter was not amplified.

The *acrAB* and *acrEF* promoters were successfully amplified for Gibson assembly. To clone these promoter regions into pCS26, they were each first amplified using PCR and run on a 1.5% agarose gel (Fig. 6). A pUC19 positive control and a negative control, which lacked template DNA, worked as expected. The gel showed bands at approximately 400 bp in both the *acrAB* and *acrEF* amplification. This suggests that the promoter regions of both operons were successfully amplified. The *ampR* promoter lane showed no band.

Ligation of pCS26 with the *acrAB* and *acrEF* promoter upstream of *luxCDABE* was successful. Gibson assembly was used to clone the PCR-amplified promoters into a XhoI and BamHI-digested pCS26. The Gibson assembly products were subsequently transformed into DH5α competent cells. The isolated plasmids were sequenced using the forward primers designed for Gibson assembly (Table 2). Sequencing confirmed that cloning successfully yielded an *acrAB*-pCS26 and an *acrEF*-pCS26 recombinant reporter vector (Fig. S1).

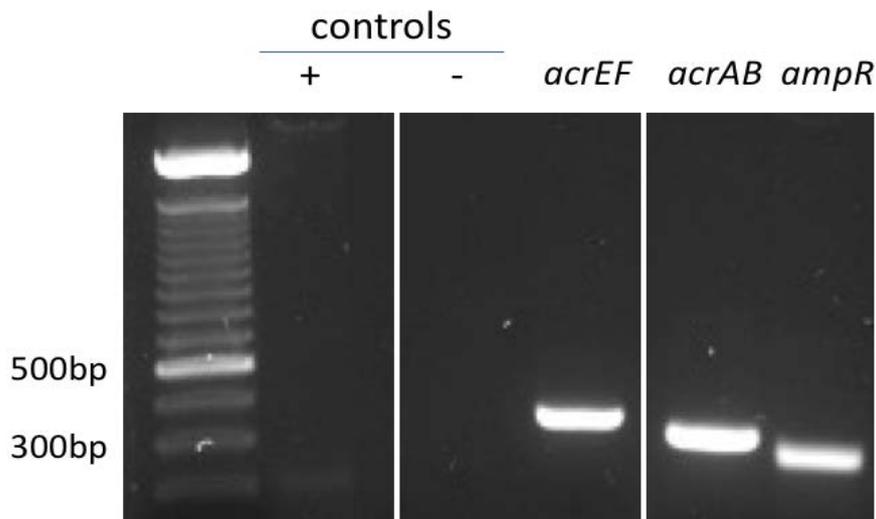


FIG. 5 The promoter regions were successfully amplified for ligation into pCR™2.1-TOPO® vector. PCR product of promoter amplification run on 1.5% agarose gel stained with SYBR® Safe DNA Gel Stain run in 1X TBE for 40 mins at 120 V. Products shown were amplified using primer set 1 (Table 1).

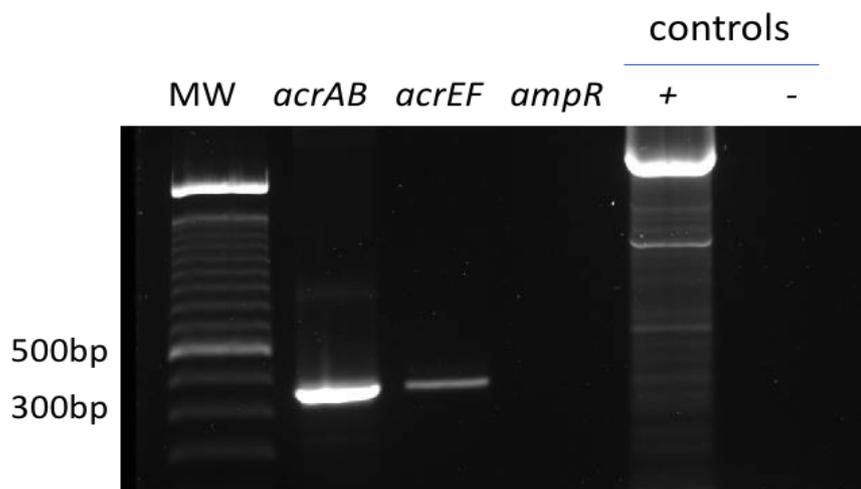


FIG. 6 The promoter regions of *acrAB* and *acrEF* were successfully amplified for Gibson assembly. PCR product of promoter amplification run on 1.5% agarose gel stained with SYBR® Safe DNA Gel Stain run in 1X TBE for 40 mins at 120 V. Products shown were amplified using primer set 2 (Table 2).

DISCUSSION

The objective of this study was to create reporter plasmids containing the *acrAB* and *acrEF* operon promoters to elucidate the control of these operons by the AcrS regulator. The *acrAB* and *acrEF* promoter regions were successfully amplified for use in two different cloning strategies. Both the *acrAB*-pCS26 and *acrEF*-pCS26 reporter plasmids were created, but the light-producing function of these constructs must still be determined.

Initial amplification of the promoter regions for insertion in TOPO. During amplification of the *acrAB*, *acrEF* and *ampR* promoter regions, the pUC19 positive control failed to show any amplified bands after gel electrophoresis. This suggests a failure of the PCR reaction, despite successful amplification of both the *acrAB* and *acrEF* promoters. This may be because the initial primer set used for amplification of the promoters required very high annealing temperatures. It is likely that the primers present in the positive control could not anneal sufficiently at this high temperature to produce a detectable quantity of amplicon DNA.

Creation of *acrAB* and *acrEF* pCS26 reporter plasmids using Gibson assembly. Primers were designed to add 40 bp of pCS26 vector sequence flanking the amplicon for subsequent Gibson assembly cloning into pCS26. The previously-created TOPO® vectors containing amplified promoters were used as template DNA in order to increase the yield of amplified products. Gibson Assembly efficiently combines DNA fragments with overlapping sequences on each end. For this reason, primers for Gibson Assembly were designed to include 40 bp derived from the pCS26 vector as well as a 20 bp sequence that was complementary to the template DNA stored in the TOPO® vector. As only the 20 bp portion of the 60 bp primer annealed to the template DNA in the early PCR cycles, the initial primer annealing temperature required for amplification was extremely low. Once amplification at this low annealing temperature is achieved, the resulting amplicons will seed the reaction for subsequent PCR cycles. As subsequent amplicons contain the full 60 bp sequence encoded by the primers, the annealing temperature is increased with each additional cycles.

Thus, to achieve successful amplification, a reverse touchdown PCR was conducted in which the annealing step temperature was initially set at 55°C and then increased incrementally by 1°C over multiple cycles. The visible bands at approximately 400 bp suggested the successful amplification of the *acrAB* and *acrEF* promoter sequences, however the *ampR* promoter failed to amplify (Fig. 6). These sequences were subsequently cloned into the pCS26 vector through Gibson assembly and transformed into DH5α for future use.

The failure to amplify the *ampR* promoter has implications for future experiments. The *ampR* promoter was intended to be a control in future luciferase assays investigating this system. This will need to be overcome in order to detect the true effects of AcrS on the expression of *acrAB* or *acrEF* as the baseline change in gene expression due to treatment must be taken into account.

Future Directions This study outlines the creation of pCS26 reporter plasmids containing the promoter regions of the *arcAB* and *acrEF* operons. In order to elucidate the role of AcrS as a possible repressor of both operons, future studies should test the light-producing function of the recombinant pCS26 vectors containing the *acrAB* and *acrEF* promoter. PCR amplification and Gibson assembly cloning should be repeated as described, in order to create a recombinant pCS26 vector that contains an *ampR* promoter which can be used as a control to provide a baseline measure of AcrS-independent light production in luciferase assays. A Δ *acrS* strain should be double transformed with both the recombinant pCS26 and one of the pMAPS vectors (8). These cells should be treated with varying concentrations of arabinose to induce AcrS expression from pMAPS. Promoter repression of *acrAB* and *acrEF* can then be measured indirectly using a luciferase assay. According to the model we have proposed, induction of AcrS expression in double transformants should lead to decreased expression of both an *acrAB* or *acrEF* promoter driven *luxCDABE* operon (Fig. 2).

ACKNOWLEDGEMENTS

This project would not have been possible without the funding from the Department of Microbiology and Immunology at the University of British Columbia. We would like to thank Dr. David Oliver for his ongoing support. His expertise and feedback were vital in the implementation and development of this project. We would also like to acknowledge our teaching assistants, Gyles Ifill and Ashley Arnold, for their valuable insight. Finally, we would like to thank Dr. Julian Davies, for generously providing us with the pCS26 vector.

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