Expression of Wild-Type rcsB Gene Under the pBAD Promoter Restores Capsule Production in Escherichia coli rcsB Mutant and Permits Normal Cell Morphology

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Previous studies have been inconclusive whether increased capsule size directly confers antibiotic resistance in Escherichia coli. In this study, rcsB gene expression was upregulated using the vector araBAD promoter and induction with varying concentrations of L-arabinose to determine the degree of rcsB involvement in determining capsule size. The rcsB gene was amplified by PCR and cloned into the pBAD-TOPO construct. Restriction digest analysis confirmed that rcsB was present in the correct orientation. Capsule visualization which followed subcloning of the constructs into an rcsB deficient E. coli strain showed restoration of a capsule, but showed little increase in size over wild-type at 2% L-arabinose induction. When the empty pBAD-TOPO vector was transformed into the same rcsB deficient E. coli strain, the L-arabinose induction yielded elongated tubular poles and additional changes in cell morphology. Normal cell morphology was observed for cells containing the pBAD-rcsB construct. Our results indicate that overexpression of rcsB gene does not greatly enhance capsule formation in the absence of a stimulant and overexpression of empty vectors would not be a suitable control for assessing the role of a cloned gene.

Colanic acid is a major component of the bacterial capsule which protects the bacterial cells from external environmental stress conditions such as changes in temperature, sub-inhibitory antibiotic treatments, and osmotic shock (1, 2). The extracellular polysaccharide structure forms an amorphous, highly hydrated gel around the bacterial cell to protect against the damaging effects of environmental stress (1).

In E. coli, the RcsF/RcsC/RcsD/RcsA-RcsB multicompartment phosphorelay system regulates the expression of more than 150 genes, including genes involved in cell division such as ftsZ and capsule production through the cps operon (1, 3, 4, 5, 6). The Rcs pathway also involves an additional cytoplasmic auxiliary protein, RcsA (3). While RcsA is known to couple with RcsB, RcsB can either act alone or in association with auxiliary regulators for regulation of the cps operon. The overexpression of RcsB is sufficient to activate the cps genes in the absence of RcsA, whereas rcsB mutants cannot express cps genes regardless of the level of RcsA (3). As such, rcsB is considered the primary regulator of the cps genes and is absolutely required for capsule synthesis (3). The binding of RcsB requires interaction with the RNA polymerase to stabilize the binding before allowing its activation (3).

Since RcsA levels are limited due to its rapid degradation by the lon protease, a recent study used a lon mutation of E. coli to increase capsule size in order to study the effects of kanamycin and streptomycin (7). They found no discernible relationship between increased capsular polysaccharide content and antibiotic resistance in E. coli K12 cells (1, 7). Since the results were dependent on the lon protease and the rcsA gene, we attempted to investigate whether capsule synthesized in the RscB-

The forward primer and reverse primer sequences are listed in Table 2.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. E. coli BW25113 and JW2205 strains were streaked on Luria-Bertani (LB) plates for whole cell PCR amplification and cloning. An additional 100 μg/ml ampicillin antibiotic supplement was added to separate agar media to test for antibiotic resistance in the wild type strains.

PCR primer design for RscB. Primers were designed to amplify the 651-nucleotide region containing the wild-type rcsB gene in E. coli BW25113 chromosomal DNA made suitable for cloning into pBAD-TOPO-TA vector system. Whole cell PCR amplification for RscB. Isolated E. coli BW25113 cells from overnight LB-agar plate culture were directly transferred and resuspended in 20 μl distilled water by
TABLE 1 Genotypes of the *E. coli* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host genotype</th>
<th>Plasmid</th>
<th>Source</th>
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<tr>
<td>BW25113</td>
<td>ΔF−, ΔaraD−araBΔ567, ΔlacZ4787:::rrnB-3, LAM−, rph−1, Δ(rhaD−rhaB)568, hsdR514</td>
<td>None</td>
<td><em>E. coli</em> Genetic Stock Centre (Yale University, New Haven, CT)</td>
</tr>
<tr>
<td>JW2205-2</td>
<td>ΔF−, ΔaraD−araBΔ567, ΔlacZ4787:::rrnB-3, LAM−, ΔrhcB770::kan, rph−1, Δ(rhaD−rhaB)568, hsdR514</td>
<td>None</td>
<td><em>E. coli</em> Genetic Stock Centre (Yale University, New Haven, CT)</td>
</tr>
<tr>
<td>CGKV12w1</td>
<td>ΔF−, ΔaraD−araBΔ567, ΔlacZ4787:::rrnB-3, LAM−, ΔrhcB770::kan, rph−1, Δ(rhaD−rhaB)568, hsdR514</td>
<td>pBAD TOPO with rcsB insert</td>
<td>This study</td>
</tr>
<tr>
<td>CGKV12w2</td>
<td>ΔF−, ΔaraD−araBΔ567, ΔlacZ4787:::rrnB-3, LAM−, ΔrhcB770::kan, rph−1, Δ(rhaD−rhaB)568, hsdR514</td>
<td>pBAD TOPO</td>
<td>This study</td>
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Picking up cells using the end of a sterile pipette tip. Samples were heated for 10 minutes at 94°C and subsequently placed into -80°C freezer for at least 10 minutes. PCR reactions consisting of 10X PCR buffer (supplied by Invitrogen kit, Cat #18038), 10 mM dNTP mix, and 10 μM of forward and reverse rcsB primers were made up to final volume of 25 μl using sterile water. Samples were thawed and centrifuged for 5 minutes at 13,200 rpm. 5 U/μl Taq polymerase (Invitrogen, Cat. #18038-042) was added just before starting the PCR reaction. PCR reactions were carried out in the Bio-Rad® Gene Cycler™ Thermal Cycler (Cat #170-6700) at 94°C for 5 minutes, followed by 35 cycles of amplification: denaturing at 94°C for 45 seconds, annealing at 55°C for 30 seconds and then elongation at 72°C for 90 seconds. For a final extension, the reaction was held for 10 minutes at 72°C, followed by a pause at 4°C until storage in the -20°C freezer.

DNA gel electrophoresis. 0.7% (w/v) or 1.0% (w/v) of agarose was dissolved in 1X TAE buffer (40 mM Tris Base (Fischer Bioreagents, Cat. #BP152-1), 20 mM glacial acetic acid (Acros, Cat. #42322-0025), 1 mM EDTA (Sigma, Cat. #E4884-500G), pH 8). A volume of 4 μl of GeneRuler™ DNA ladder (Fermentas, Cat. #SM0312) with 1X DNA loading dye was used to prepare the molecular weight standard. Prior to being loaded into the wells, sample DNA loading dye was added.

**pBAD-TOPO plasmid cloning reaction.** The amplified rcsB PCR product (4 μl) was cloned into the pBAD-TOPO plasmid vector with an L-arabinose-inducible araBAD promoter (P<sub>araBAD</sub>), as outlined by manufacturer’s instructions using the pBAD TOPO TA® Expression Kit (Invitrogen, Cat. #K43000-01). The resulting plasmid containing the ligated PCR product along with a positive control (vector without insert) were transformed into One Shot<sup>®</sup> TOP10 chemically competent *E. coli* cells and incubated on ice for 3 minutes before heat-shocked in a 42°C water bath for 30 seconds. 250 μl of super optimal broth with catabolite repression (SOC) medium was added to all transformants and incubated at 37°C on a shaking platform for 200 rpm for 1 hour. Transformants were subsequently plated onto pre-warmed Luria-Bertani (LB) + 100 μg/ml ampicillin agar plates and incubated at 37°C overnight.

**pBAD vector isolation.** Plasmid isolation was carried out according to the manufacturer’s instructions on ten overnight cultures of One Shot<sup>®</sup> TOP10 transformed *E. coli* cells in 10 ml LB/Amp broth using the GeneJET<sup>®</sup> Plasmid Miniprep Kit (Thermo Scientific, Cat. #K0502). PCR analysis using the forward and reverse rcsB designed primers was performed to confirm an insert. Touchdown PCR was carried out on the isolated rcsB-pBAD vector using the Bio-Rad® Gene Cycler™ Thermal Cycler (Cat #170-6700): denaturing at 94°C for 45 seconds, annealing at 69°C, decreased 1°C during each cycle until 54°C, followed by elongation at 72°C. Subsequent cycles were repeated at 54°C annealing temperature for a total of 35 cycles. The Nanodrop 2000 (Thermo Scientific) was used to determine the A<sub>260</sub> and A<sub>280</sub> values in order to assess plasmid purity and quantities.

**Plasmid insert orientation analysis.** BstEII restriction digests were performed on the isolated plasmids using 1X NE buffer 3 (NEB, #B7003S), 1X purified BSA (NEB, #B9001S), 0.25 g of plasmid DNA, 5 U of BstEII (NEB, #R01625), and sterile water to make up to a final volume of 20 μl. A reaction with Lambda DNA was included as a positive control to confirm the actions of BstEII. The digests were mixed by pipetting and centrifuged a short round before incubation at 60°C for 1 hour.

**Subcloning into *E. coli* JW2205-2 strain.** Using results from the restriction digest analysis, an overnight culture of a successfully transformed pBAD-rcsB strain was prepared for plasmid isolation using the GeneJET<sup>®</sup> Plasmid Miniprep Kit. Competent *E. coli* JW2205-2 cells were prepared using the Hanahan Protocol with MgCl<sub>2</sub>–CaCl<sub>2</sub> chemical treatments, as outlined by Sambrook and Russell (8). The isolated plasmid DNA was transformed into *E. coli* JW2205-2, a ΔrcsB770::kan mutant strain. A negative control (pBAD-TOPO vector, no insert) was also transformed into the mutant JW2205-2 cells. Sub-cloned transformants were incubated on ice for 25 minutes before heat-shocked in 42°C water bath for 30 seconds. 800 μl SOC medium was added to all transformants and incubated at 37°C on a shaking platform at 200 rpm.

**TABLE 2** rcsB primer sequences designed for amplification of wild type rcsB for ligation into pBAD TOPO.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5’ to 3’)</th>
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<tr>
<td>TOPO rcsB-f-3’-gamma (forward rcsB)</td>
<td>TTAGCTCTTAATCGCGGACTAAAGTCAC</td>
</tr>
<tr>
<td>TOPO rcsB-r-3’-gamma (reverse rcsB)</td>
<td>TGAGAGGACTTGCTAATGAACTGACGTA</td>
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</table>

Underlined sequences are complementary to native rcsB sequence. Italized sequences indicates the ribosome binding site. Non-underlined sequences indicate required nucleotide spacers within pBAD-TOPO cloning site to put sequences in-frame.
To determine if the colonies prepared in the overnight prior to being plated on was prepared in the evening, the concentration at which there is an absence of bacterial growth. Minimum inhibitory concentration was determined by looking for the lowest antibiotic concentration to make the desired final antibiotic concentrations of the tetracyclines, chloramphenicol, streptomycin) combination to make the desired final antibiotic concentrations of 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 μg/ml in 100 μl of MH medium supplemented with 2% L-arabinose. Then, 100 μl of diluted culture was added to each well. The plates were incubated at 37°C in the dark for 24 hours. Minimum inhibitory concentration assay. E. coli BW25113, JW2205-2, CGKV12w1, CGKV12w2 strains were streaked on LB plates supplemented with 0.002%, 0.02%, 0.2%, 0.5%, 1.0% and 2.0% L-arabinose (Sigma-Aldrich, Cat. #A91906-100G-A), and grown at 37°C overnight prior to being stained. LB plates without arabinose were also grown for each strain as the negative control to compare the effects of arabinose. The technique for staining is as outlined by Chiang et al (9). A 1% Congo red (Sigma Cat. #6767) solution was prepared in sterile water. Maneval’s stain was prepared by combining 15 ml of 5% aqueous phenol (Fisher Cat. #108-95-2), 15 ml of 20% aqueous glacial acetic acid (Acros, Cat. #42322-0025), 2 ml of 30% aqueous ferric chloride (Fisher #7705-08-0), and 0.75 ml of 1% aqueous fuchsine (Baker #232-2119). For capsule staining, a drop of 1% Congo red solution mixed with a loopful of 1% aqueous acid fuchsin (Baker #232-2119) was applied to the smear and backed into the mixture to allow for a thin smear. An angle and backed into the mixture to allow for a thin smear. Subsequently, a few drops of Maneval’s stain was spread using the same method mentioned for the Congo red staining. After incubation at room temperature for 60 seconds, the slides were rinsed with distilled water and blotted dry with a paper towel. The samples were visualized with immersion oil under 1000X magnification with a Leitz Diaplan light microscope and photographed (Leitz, Wetzlar, Germany).

Minimum inhibitory concentration assay. E. coli BW25113, JW2205-2, CGKV12w1, CGKV12w2 strains were grown overnight in LB broth and diluted to an OD_600 reading of 0.4. The assay was performed in Sarstedt sterile 96-well plates. Two fold dilution series were made in duplicate for each strain and antibiotic (tetracycline, chloramphenicol, streptomycin) combination to make the desired final antibiotic concentrations of 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 μg/ml in 100 μl of MH medium supplemented with 2% L-arabinose. Then, 100 μl of diluted culture was added to each well. The plates were incubated at 37°C in the dark for 24 hours. Minimum inhibitory concentration was determined by looking for the lowest antibiotic concentration at which there is an absence of bacterial growth.

RESULTS

Whole cell PCR amplification of rcsB yielded expected bands at 651 bp. PCR amplification of rcsB from E. coli BW25113 showed a single bright band between 750 bp and 500 bp on the DNA ladder marker table (Fig. 1). This band corresponds to the expected 651 bp fragment size of the rcsB gene. Nonspecific activity of primers amplified a very faint band fragment of approximately 900 bp but the 651 bp fragments were present in a much greater quantity.

Transformation of chemically competent TOP10 E. coli. Following the cloning of rcsB gene into the pBAD TOPO vector, transformants were selected by growing onto LB/Amp agar plates. A positive control was prepared by transforming One Shot® TOP10 chemically competent E. coli cells with empty pBAD TOPO vector to assess whether the vector was functional in conferring ampicillin resistance to the cell and whether the vector by itself had an effect on cell physiology. Reasonable growth of colonies in both the transformants and control samples suggested that both the ampicillin resistance and transformation reaction were efficient.

Restriction digest analysis confirmed correct rcsB insert orientation in two transformants. To determine whether the transformants carried the pBAD TOPO vector with the rcsB insert in correct orientation, a restriction digest was conducted on isolated plasmids. Restriction enzyme BstEII was chosen since pBAD TOPO vector has single BstEII site at position 764 bp downstream from the 5’end and in the rcsB PCR gene product at 516 bp. The expected sizes from successful ligation are 4126 bp for the pBAD TOPO vector and 4777 bp for the pBAD-rcsB construct. Two expected band sizes of around 850 bp and 3900 bp were observed in lanes 7 and 8 of Fig. 2. This verified that these competent TOP10 E. coli strains have been transformed with pBAD-rcsB construct. The molecular weight of a single distinct band size observed in both lanes 5 and 6 is consistent with the size of linearized pBAD TOPO vector and therefore indicates no insertion of the gene. As expected, uncut closed-circular form of pBAD TOPO vector in lane 4 migrated slowly than a
It is interesting to note that while our rcsB mutant strain, the RcsB, leading to low cps expression (2, 3, 4, 5). RcsC, a sensor kinase in the phosphorelay system becomes autophosphorylated upon external stimuli, and then transfers phosphoryl groups to downstream RcsD and then RcsB (2, 3, 5); and it is this phosphorylated form of RcsB that acts as a positive regulator for colanic acid synthesis. A limitation to our experiment was that an environmental stimuli such as changes in the temperature or osmolarity was not introduced to our transformed mutant cells. Therefore, the similarity in the quantity of capsule between wild-type and our transformed mutant as observed in Fig. 4, may have been a result of higher levels of RcsB in its unphosphorylated inactive form (12). In addition to the absence of an activating signal, increased phosphatase activity of RcsC would result in the net dephosphorylation of phosphorylated-RcsB, leading to low cps expression (12). In other words, any RcsB proteins that may have phosphorylated through RcsC-independent pathway can still undergo dephosphorylation by RcsC in the absence of stimuli. These observations mean that overexpression of rcsB gene in the absence of stimuli is upon induction with arabinose resulted in cell morphologies that were indicative of fault with the cell division mechanisms (Fig. 4D). These cells had elongated and enlarged morphologies up to 30 times that of an average cell. The cells seen in Fig. 4D can have a variety of shapes, but a majority of cells can be described to have a lemon shape with one or two tubular poles. Further observations revealed that the lengths of these tubular poles to be dependent on the time of incubation with longer incubation times resulting in a longer poles and larger cell centre (data not shown). Induction of CGKV12w1 with increasing arabinose concentrations did not showed a correlated increase in capsular size as expected (data not shown). However, induction with 2% arabinose shows capsule production that had a slight increase in size over the wild-type strain.

**DISCUSSION**

We have constructed an rcsB upregulated strain by cloning the wild-type gene from *Escherichia coli* BW25113 into the pBAD vector which allows for controlled induction by L-arabinose. Although protein levels of RcsB have not been confirmed quantitatively, we were able to observe a difference between capsule sizes by 2% L-arabinose induction and no induction. The observable change in capsular size is indicative of rcsB gene expression.

While the presence of amplified rcsB gene in the ΔrcsB strain was indicative of a successful transformation, we were unable to observe significant changes in the capsule size as we initially expected. Unlike the previous study done by Drayon *et al.* where there was a substantial increase in the capsule size of the *lon* mutant strain, the overexpression of rcsB gene seemed to have only small effect on capsule synthesis (7). This outcome was unexpected since it is a known observation that *E. coli* synthesizes colanic acid polysaccharide capsule through the RcsCDB phosphorelay system, in which RcsB is a key transcriptional activator of the *cps* gene (2, 3, 4, 5).

**Single cut, linearized form.** Lastly, fragmentation of λ DNA indicates that restriction digestion activity of *BstEII* was optimal at 60°C.

**Whole cell PCR of rcsB insert in subcloned *E. coli* JW2205-2.** Presence of a single band with molecular weight of 651 bp in Fig. 3A confirms that the *E. coli* JW2205-2 strain has been successfully transformed with plasmid carrying rcsB gene. The comparison was obvious as the JW2205-2 strain prior to subcloning did not amplify any rcsB gene, while wild type *E. coli* BW25113 successfully amplified rcsB gene as observed in Fig. 3B. Since this particular JW2205-2 strain has rcsB gene knocked out, no normal cellular rcsB gene was expected. These negative and positive controls confirm that the band observed in Fig. 3A is a result of transformation with pBAD-rcsB vector.

**Transformed vector containing rcsB gene increased capsule size of *E. coli* rcsB mutant strain following arabinose induction.** To examine the effect of increasing levels of rcsB expression we compared the cell morphologies of wild-type *E. coli* (BW25113) with *E. coli* strains thought to underproduce and overproduce capsule. We grew transformed mutant strains containing pBAD-rcsB, mutant strains containing the vector alone in the absence or presence of increasing 0.002%, 0.02%, 0.2%, 1% and 2% L-arabinose. When grown in the absence of arabinose, we observed a moderate size of capsule around the wild-type and no obvious capsule surrounding the cell body of the rcsB mutant strain (JW2205-2) (Fig. 4A and 4B respectively). It is interesting to note that while our transformed pBAD-rcsB mutant strain (CGKV12w1) produced no capsule in the absence of arabinose, the strain with the empty vector produced a capsule of noticeable size in the presence and absence of an arabinose inducer (Fig. 4E and 4C respectively). Furthermore, CGKV12w2 was optimal at 60°C.
insufficient to account for its maximal function, which is to bind to and activate cps gene expression.

The pBAD-TOPO empty vector control transformed into the E. coli JW2205-2 mutant presented unexpected and unusual results (Fig. 4D). Cell morphological observations revealed elongated tubular poles running across the short axis of the cell, suggesting that cell division mechanisms may be at fault. A similar observation had been documented in a paper by Varma et al. dealing with the simultaneous inhibition of FtsZ and MreB in penicillin binding protein mutants of E. coli (10). Their findings revealed that FtsZ may be responsible for septation and a required component in directing the insertion of new peptidoglycan into the lateral walls of E. coli (10). In addition, they found that the uneven shape of the cell suggests that new peptidoglycan was deposited unevenly over the entire surface of the cell (10).

Although the unevenness or lemon-shaped morphology seen in our E. coli is consistent with the descriptions documented by Varma et al., Fig. 4D had significant differences in both the starting conditions and morphology. In particular, the elongation of the poles, while still retaining a lemon shape, were significantly longer in length than the sizes documented by Varma et al. Furthermore, the requirements to produce an altered morphology as seen in Fig. 4, seemed to be dependent on the combination of all three starting factors: a rcsB gene deletion, empty pBAD-TOPO vector, and L-arabinose. Another paper studying the morphologies of cells involving similar starting conditions, but with a csrA mutant produced no altered morphologies when compared with wild-type strains (11). In E. coli, the RcsBC two-component system has been documented to be in charge of regulating the cell division gene ftsZ through rcsB-dependent stimulation (1, 3, 5). However, the differences seen in our paper requiring arabinose and empty pBAD-TOPO vector may imply new interactions between rcsB and FtsZ-MreB directed mechanisms beyond what has been previously documented.

In conclusion, the pBAD-rcsB construct was able to upregulate the capsule size in rcsB knockout E. coli strains above the wild-type phenotype. However, this increase was insufficient to conclude that we have successfully expressed the RcsB protein and therefore activate cps transcription. Additionally, we observed altered cell morphology in CGKV12w-2 cells under the induction of L-arabinose, which shows that an empty vector is not a suitable control for assessing the role of a cloned gene.

**FUTURE DIRECTIONS**

Although we have constructed an RcsB upregulated strain of E. coli, the amount of upregulation was minimal and insufficient to determine whether the rcsB-dependent pathway confers antibiotic resistance. Future work should test for the effect of a stimulus that can elicit a better capsule response. The level of rcsB expression should also be quantitatively measured by isolating rcsB mRNA and performing real-time PCR to obtain cDNA. Then, antibiotic resistance can be determined by using a wide range MIC assay and a correlation can be made between the two variables.

Our results suggest that it is the pBAD-TOPO vector that is the cause of the altered cell morphology. To confirm if the pBAD-TOPO vector itself is the cause of interrupted cell division, RNA interference may be performed to silence the rcsB gene in CGKV12w-1 cells. However, future research teams should focus on determining why rcsB prevented the altered phenotype in Figures 4C and 4D. Since rcsB is known to regulate the ftsZ gene for cell division, it would be useful to determine if this precise interaction is the sole cause of the altered phenotype. For example, the morphology of E. coli strains with mutations in the ftsZ gene carrying pBAD-TOPO clones could be compared to that of their respective controls of empty pBAD-TOPO vector transformed into the same mutant strain. If similar cell morphologies are also observed in these mutant strains carrying the empty vector, then the phenotype may be narrowed down to the RcsB-ftsZ gene interaction.

Alternatively, other E. coli mutants should be used to assess whether there is a general suppression of the altered
phenotype or whether it is limited to rcsB mutants. If altered cell morphologies are also observed in other mutant strains carrying the empty vector, then the phenotype is most likely due to a direct effect by the vector itself. In addition, the region of the plasmid downstream of the TOPO cloning site should also be examined carefully to assess its role in causing the observed phenotype.

Furthermore, the strains should be grown for longer periods of time and observed at different time intervals with Maneval’s staining to give insight on timing of cell division, which is essential for culture growth.

ACKNOWLEDGEMENTS

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REFERENCES