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Determining the Relationship Between the *lac* Operon and the Expression of *brkA* on pDO6935 in *Escherichia coli* Using a Promoterless Green Fluorescent Protein Reporter Gene

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SUMMARY The *lac* operon regulatory system in *Escherichia coli* plays a role in controlling gene expression through transcriptional regulation. Understanding the regulation of specific genes is essential for elucidating their roles in bacterial physiology and pathogenesis. We chose to investigate green fluorescent protein (GFP) as a proxy for BrkA, an autotransporter protein in *Bordetella pertussis* responsible for serum resistance and host cell adherence. In this study, we aimed to introduce an additional copy of *lacI* into the system to establish a tighter regulation, as well as deleting the promoter region of the *lac* operon of *E. coli* to investigate its effects on downstream GFP expression as a proxy for *brkA* expression. Isopropyl β-d-thiogalactoside (IPTG)-controlled GFP expression was demonstrated as an indicator of operon activity, and an increased GFP expression was observed upon IPTG induction, indicating a tighter regulation of GFP, further reinforcing the regulatory role of the *lac* operon on the downstream expression of GFP. Due to time restraints, the deletion of *lac* promoter region did not work out as planned, suggesting a room for us to reflect and troubleshoot. Our findings highlight the importance of the *lac* operon and its utility in studying BrkA expression in *E. coli*.

INTRODUCTION

BrkA is an autotransporter protein found in *Bordetella pertussis* which is involved in serum resistance and adherence (1). Interestingly, BrkA was found to be a potential therapeutic target (1). Thus, efforts to study expression of BrkA in *Escherichia coli* made through plasmid constructs like pDO6935 clinically valuable (2). In *B. pertussis*, *Bordetella* virulence gene drives *brkA* expression (2). Yet, the promoter regulating *brkA* expression in pDO6935 remains unclear. Using a promoterless reporter plasmid containing green fluorescent protein (GFP), Biju et al. suggested that *brkA* expression was being driven by the *lac* operon in pDO6935 (3).

The *lac* operon, which is nearly ubiquitous in *E. coli*, encodes the ability to transport and metabolize lactose (4). It is comprised of three structural genes: *lacZ* encodes a β-galactosidase that splits lactose into glucose and galactose; *lacY* encodes a β-galactosidase permease which helps transport lactose into the cell; and *lacA* encodes a galactoside transacetylase (5). Expression of these genes benefit the cell when lactose is the best carbon source available. Thus, the *lac* operon is positively regulated by lactose, and negatively by glucose through the activity of transcriptional factors (6). Cyclic adenosine monophosphate (cAMP) receptor protein (cAMP-CRP) regulator complex is another factor that affects *lac* operon activity. It promotes the recruitment of RNA polymerases upstream of the *lac* promoter (7). cAMP production is reduced in the presence of glucose, lowering access to the cAMP-CRP complex (6). In the absence of lactose derivatives or artificial inducers like isopropyl β-d-thiogalactoside (IPTG), LacI binds the *lac* operator, repressing transcription (8).

However, Biju et al. found that under IPTG treatment, GFP expression did not increase as expected (3). This reason might be that the *lac* operator may have stoichiometrically outnumbered the LacI repressor which led to leaky expression of GFP regardless of the presence of IPTG. Additionally, a study performed by Haniak et al. created novel vectors from pDO6935, one of which introduced GFP within the lac regulatory system upstream of

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brkA (9). Taken together, further focusing on how GFP expression — a proxy for *brkA* expression — in pEAH23B made by Haniak et al. would better elucidate the role of the *lac* operon regarding *brkA* expression.

In this study, we aim to further address the uncertainty of the role of the *lac* operon in *brkA* expression, to see if i) the introduction of one extra *lacI* leads to tighter regulation of the expression of GFP or ii) the removal of promoter region in the *lac* operon inhibits downstream GFP expression. By using the term "tighter", we aim to achieve a more significant change in GFP expression with respect to IPTG concentrations.

To achieve this, firstly, we will introduce pEAH23B into *E. coli* strain BL21 (DE3) containing an additional copy of *lacI* to see if there is establishment of a tighter regulation (8). Secondly, our goal is to disrupt the *lac* regulatory system by mutagenizing the promoter region, creating a complete deletion of the promoter region. We hypothesize that i) the introduction of an additional *lacI* will give tighter regulation, and ii) if *brkA* is regulated by the *lac* operon, its expression will significantly decrease in the absence of the promoter.

METHODS AND MATERIALS

Bacterial strains and plasmids. All bacterial strains including pSB1C3 and pEAH23B were acquired from the inventory of the Microbiology & Immunology Department at the University of British Columbia (UBC). The pSB1C3 vector with no promoter was obtained from Dr. Avery Noonan (Hallam lab, UBC), and the pEAH23B vector was constructed by Haniak et al. (9).

Primer design. Forward and reverse primers were created for PCR and Q5 site-directed mutagenesis by selecting sequences flanking the promoter region of the *lac* operon of the template pEAH23B on Snapgene software. Sequences and melting temperatures are indicated in Supplemental Table S1. Primers were obtained from Integrated DNA Technologies.

Luria Bertani (LB) broth and chloramphenicol (CAM) media preparation. LB broth was prepared by adding 10 g tryptone, 5 g yeast extract, and 10 g NaCl to 1 L of distilled water (10). After mixing the components into a homogenous mixture. For LB agar plates, 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g agar were added to 1 L of distilled water. For LB broth and agar plates with CAM, CAM was added to a final concentration of 25 µg/mL prior to use (10).

Overnight culture preparation. Cultures of *E. coli* BL21 were grown in 10mL of LB media and incubated overnight at 37°C with shaking. 1µL of CAM was added to each tube when *E. coli* BL21 was transformed with plasmid pSB1C3 and pEAH23B.

Plasmid extraction and quantification. Plasmid pSB1C3 and pEAH23B were isolated using the EZ-10 Spin Column Plasmid DNA Miniprep Kit (BioBasic). The quantification and evaluation of DNA concentration and purity were performed using a NanoDrop2000® Spectrophotometer (ThermoFisher) at 260 nm.

Agarose gel electrophoresis. 1% agarose gels were created using 1 g of agarose and 100 mL of 1X TAE buffer. 4 µL of SYBR Safe DNA Gel Stain was mixed in after dissolved agarose cooled down to approximately 60°C. Gel was left to solidify for 30 minutes, and 6X B7021S NEB blue loading dye was diluted to 1X using sample DNA. Thermofisher O'generuler DNA Ladder Mix was loaded to quantify band size after electrophoresis. Gel electrophoresis proceeded at 110 V until the loading dye had crossed fifty percent of the gel.

Competent cell preparation. Overnight culture was inoculated into sterile LB and placed in a shaking incubator at 37°C until OD600 reached 0.3-0.5. Cell culture was centrifuged at 4°C for 10 minutes and supernatant was discarded. Cell pellets were resuspended in 100 mM $CaCl_2$ and chilled on ice for 15 minutes. Washed cells were centrifuged at 4°C for 10 minutes and supernatant was discarded. Cells were then resuspended in ice-cold 100 mM $CaCl_2$ with 15% v/v glycerol, aliquoted, and stored at -70°C (11).

Transformation of plasmids pSB1C3, pEAH23B, and mutated pEAH23B. pSB1C3 and pEAH23B were directly transformed into *E. coli* BL21, while mutated pEAH23B was transformed into endonuclease-negative DH5α *E. coli* before being extracted and transformed into endonuclease-positive *E. coli* BL21 to amplify the mutated product. Extracted plasmids pSB1C3 and pEAH23B were added to competent cells and mixed by gentle agitation through five inversions. Mixtures were chilled on ice for 30 minutes, and heat shocked at 42°C for 30 seconds. Pre-warmed LB was added to the mixture and incubated at 37°C for 60 minutes with shaking (11).

Fluorescence agar plate and IPTG plate induction. Following transformation, isolated colonies were streaked onto two LB agar plates with 25 μg/mL CAM and the addition of 0, 40 μM, and 400 μM IPTG (New England Biolabs). Plates were streaked with E. coli BL21 transformed with plasmid pEAH23B, as well as *E. coli* BL21 strains as negative control, and *E. coli* BL21 transformed with plasmid pSB1C3 as positive control. Untransformed groups were grown on LB agar, while the transformed groups were grown on CAM25 agar. Plates were incubated overnight at 37°C and visualized using the Bio-Rad ChemiDoc imaging system with Alexa 546 settings to observe GFP expression (12).

IPTG broth induction and GFP fluorescence quantification. For IPTG broth induction, 200 μL reactions were set up in a black-walled 96-well plate. IPTG was added to a final concentration of 40 and 400 μM in LB with 25 μg/mL of CAM (CAM25). 10 μL of *E. coli* BL21 overnight culture transformed with pEAH23B was added into CAM25 media with IPTG concentrations of 0, 40 and 400 μM (12). 10 μL of *E. coli* BL21 overnight culture transformed with pSB1C3 was added into 190 μL of CAM25 media and was used as a positive control. Another 10 μL of untransformed *E. coli* BL21 was added to 190 μL of LB as a negative control. The OD_{600} and GFP (λ_{ex} = 485 nm and λ_{em} = 528 nm) of all groups were measured in the microplate reader (BioTek) for 24 hours, and data was collected every 20 mins. To normalize GFP readings, each reading was divided by its OD_{600} value for each well. Two-sample T-test was performed to calculate statistical significance using Microsoft Excel™, which was used to tabulate data and generate graphs.

Polymerase chain reaction (PCR) and Q5 Site-Directed Mutagenesis. 25-μL reactions were set up with the Q5 Hot Start High-Fidelity 2X Master Mix 10 μM Forward Primer, 10 μM Reverse Primer, template DNA (~25 ng/μl pEAH23B), and sterile distilled H_2O. The reaction was run in the Bio-Rad T100 thermocycler according to the New England Biolabs protocol and an annealing temperature of 54°C was used. The resulting PCR product was combined with 2X KLD Reaction Buffer, 10X KLD Enzyme Mix, and sterile distilled H_2O according to the New England Biolabs Q5® Site-Directed Mutagenesis Kit Protocol (13).

RESULTS

Upon IPTG induction on agar plates, we found that despite the observable reduction in fluorescence intensity on the plate lacking IPTG, there was no quantifiable data for us to draw conclusions on the significance of adding various concentrations of IPTG with respect to their fluorescence intensity (Figure 1A).

Therefore, to obtain quantifiable fluorescence data, we conducted a liquid IPTG induction assay. For visualization purposes, a curve depicting the normalized GFP readings during incubation was plotted. The normalized growth curve represented by GFP readings shows the growth of *E. coli* BL21 in different IPTG conditions (Figure 1B). We selected the last readings at 24 hours to create a bar plot for comparison between groups with various IPTG concentrations. The wells with 40 μM IPTG showed the highest level of GFP expression upon IPTG induction, with a significant increase in GFP expression ($p = 0.0007$) compared with wells without IPTG (Figure 1C). In contrast, the response to 400 μM IPTG did not reach statistical significance, which is not consistent with the results of 40 μM IPTG (Figure 1C). These results suggest that while GFP expression is regulated by IPTG in *E. coli* BL21 (DE3) with pEAH23B, it does not conclusively demonstrate tighter regulation under the tested conditions.

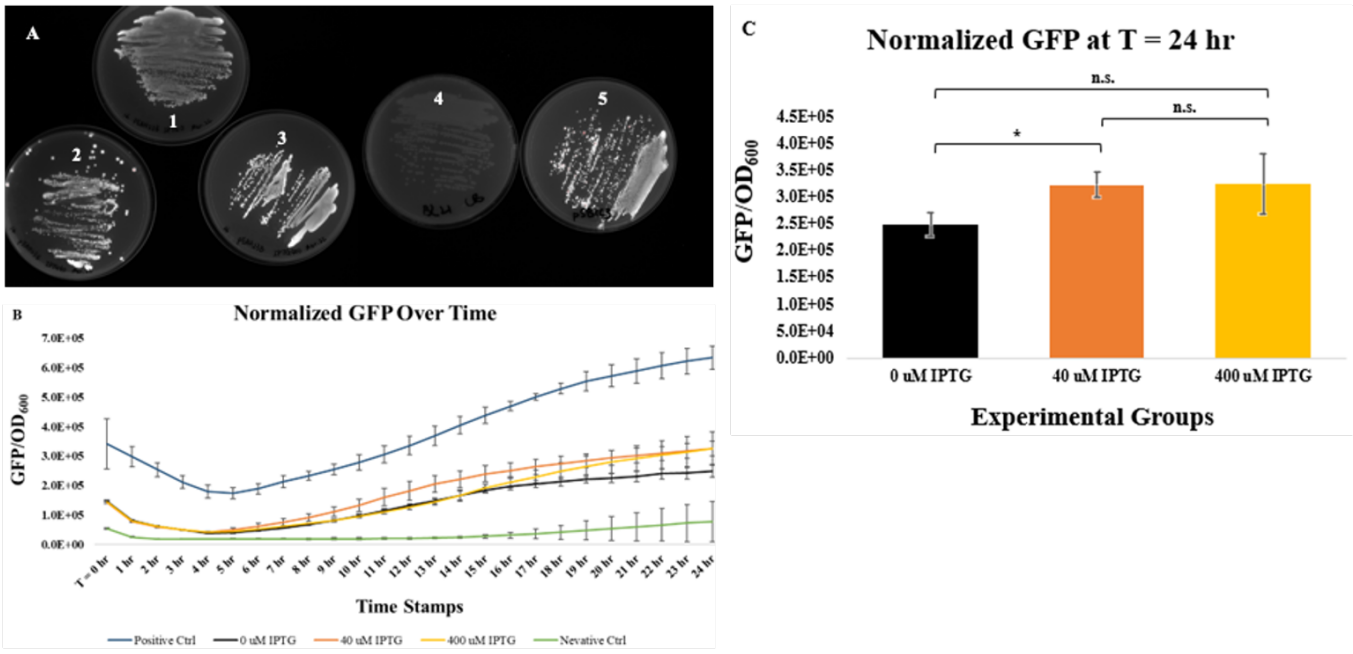

FIG. 1 GFP expression increases with increasing IPTG concentration. (**A**) LB agar plates with 25 µg/mL CAM and different concentrations of IPTG (0 µM, 40 µM and 400 µM). Plates were streaked with *E. coli* BL21 transformed with plasmid pEAH23B onto plates 1, 2, and 3, respectively. Negative control, *E. coli* BL21, was streaked on LB agar plates without IPTG or CAM (plate 4). The positive control, *E. coli* BL21 transformed with plasmid pSB1C3, was streaked on CAM25 agar (plate 5). (**B**) Quantification of GFP readings normalized to OD_{600} readings of BL21 strain transformed with pEAH23B in 200 µL LB media with 25 µg/mL CAM and 0, 40, and 400 µM IPTG after 24 hours of incubation. The negative control was LB medium with *E. coli* BL21. The positive control was CAM25 with pSB1C3 transformants culture. Statistical significance was calculated using T-test. n.s. = not significant; * indicates $p < 0.05$. (**C**) Curves showing changes of normalized GFP readings within 24 hours across the different IPTG conditions.

DISCUSSION

In this study, we aimed to first evaluate the GFP expression in the *E. coli* strain BL21 (DE3) with two copies of *lacI*, under both IPTG-stimulated and control conditions after transformation of plasmid pEAH23B. In addition, we aimed to carry out Q5 site-directed mutagenesis of the *lac* operon on pEAH23B, which did not yield plasmids according to gel electrophoresis results (Figure 2).

Quantitative analysis of GFP expression with IPTG induction showed varying results. Significantly increased GFP expression was observed in wells containing 40 µM IPTG compared to those without IPTG (Figure 1B), meaning that the introduction of one extra copy of *lacI* indeed brings tighter regulation of GFP expression.

In contrast, the expression levels in wells with 400 µM IPTG did not achieve statistical significance. This variation may be attributed to an outlier in the 400 µM IPTG condition, where one replicate exhibited considerably lower GFP expression relative to the other two replicates. Furthermore, a comparison of GFP expression between the wells with 40 µM and 400 µM IPTG suggested minimal difference in expression levels between these two conditions. These findings indicate that 40 µM IPTG may be reaching the saturation concentrations for GFP expression (i.e. the cells will no longer exhibit a higher GFP expressivity even when the IPTG concentration further increases) under experimental conditions. It was also documented in a previous research paper where much lower concentrations of IPTG were used for IPTG induction assays (14).

Despite several attempts, none of the plasmids were successfully transformed into *E. coli* DH5α after Q5 mutagenesis, due to a critical failure in the PCR step. Upon reflection, some of the issues that may lead a none-productive PCR may include incorrect annealing temperature, incorrect thermocycler programming, a complex (GC-rich) template (15). Therefore, multiple different strategies were employed to solve the potential problems

mentioned above, including altering the annealing temperature, changing the incubation time for elongation step during PCR, adding 2% dimethyl sulfoxide (DMSO) into the reaction mixture.

In addition, our agarose gel electrophoresis with the pEAH23B plasmids extracted from *E. coli* BL21 starter strains provided by the MBIM department showed truncated lengths at around 2.2 kilobases, whereas the expected length of the plasmid should be over 3 kilobases, as indicated in Figure 2 (9). This indicates that there may be some fundamental issues with the quality of pEAH23B plasmids that require further investigation including sequencing to confirm its identity.

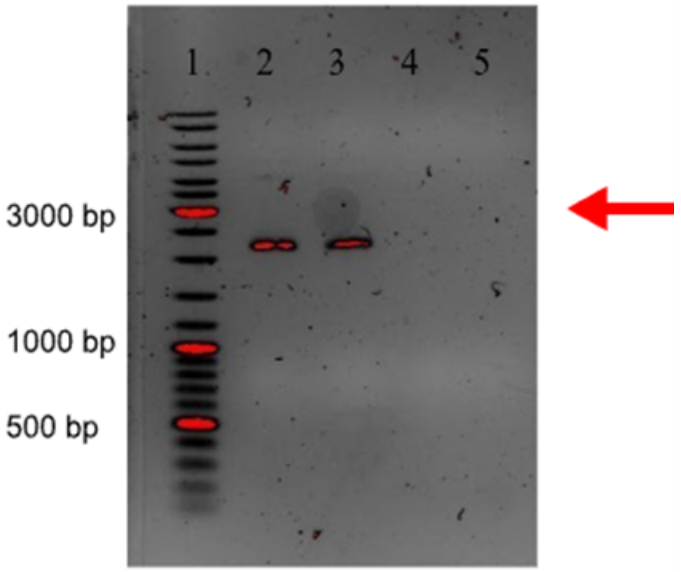

FIG. 2 Agarose gel electrophoresis of Q5 PCR amplified product and pEAH23B. Lane 1, Thermofisher O'GeneRuler DNA Ladder Mix. **Lane 2-3**, 1 ng pEAH23B. **Lane 4-5**, 1ng Q5 PCR 29 product. A red band indicates that the fluorescence of the band has reached saturation. The length of the three bands from the ladder is labeled on the left. Red Arrow indicates the position of the desired Q5 mutagenized product (3221 bp).

Conclusions In this study, we conducted experiments to modify the *lac* regulatory mechanism of *E. coli* and investigated the effects on downstream GFP expression as a proxy for *brkA* expression. We transformed *E. coli* BL21 containing an additional copy of the *lacI* repressor of the *lac* operon with pEAH23B, a modified pDO6935, in order to create a higher stoichiometric ratio of LacI repressor and its binding lac operator. We successfully demonstrated IPTG-controlled GFP expression in pEAH23B-transformed *E. coli* BL21 and have demonstrated that the extra copy of *lacI* indeed provides tighter regulation in terms of GFP expressivity. These findings provide further insight into the relationship between the *lac* operon and *brkA* expression in pDO6935 with the use of IPTG as a method of effectively regulating protein expressivity. In our second approach, we aimed to mutate the *lac* operon promoter and investigate GFP expression in an environment where *lac* operon activity is disrupted or completely shut down. Our attempts at Q5 site-directed mutagenesis to investigate the effects of deleting the promoter region were unsuccessful, and this experimental process likely requires a different approach for future related experiments.

Future Directions Despite our efforts noted above, we remain unsure if the extra copies of *lacI* repressor provide tighter regulation of GFP, and therefore *brkA* as a result. Future studies could compare GFP expressivity results of *E. coli* BL21 and a different strain only containing one copy of LacI to determine if the additional copies truly result in a tighter regulation of protein expression. Alternatively, other strains or genetically engineered BL21 strains with more than two *lacI* genes could be utilized for examination of a tighter regulation. Likewise, we noted that although the results at 40 μM IPTG are statistically significant, this is not the case at 400 μM. Future studies could perform a similar experiment with a more varied range of IPTG concentrations, prompting further investigation and potentially highlighting the importance of a mechanism that prevents higher concentrations of IPTG from being effective.

In future attempts to delete the *lac* promoter region, several factors should be considered. Firstly, the primers used should be evaluated through sequencing to ensure their authenticity. Additionally, the reverse primer used in this study appears to have the potential to dimerize,

which necessitates quantification of the size of the primers using an agarose gel. Further experimentation with different PCR master mix compositions may increase the likelihood that site-directed mutagenesis will occur. This includes testing higher concentrations of DMSO or increasing concentrations of the added primers. In addition, sequencing should be carried out for the pEAH23B plasmids to identify the removal of its *lac* promoter region upon Q5 mutagenesis.

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CONTRIBUTIONS

The project was originally conceived by RZ. All authors contributed toward refining the experimental procedure and were equally involved in experimentation. SC wrote the abstract, conclusion, future directions, and part of the materials/methods. QG wrote part of the materials/methods and took the lead in writing the results and discussion sections. MQ wrote the introduction and contributed toward the materials/methods and future directions. RZ wrote part of the materials/methods, created figures and their corresponding captions, and organized the references. All authors were involved in contributing to all sections of the manuscript during the revision process.

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