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Mapping the *brkA* Promoter Region on pDO6935 in *Escherichia coli* Using pLISA, A Novel Promoterless GFP Reporter Plasmid

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SUMMARY *Bordetella* resistance to killing A (*brkA*) is a serum resistance gene encoding an autotransporter native to *Bordetella pertussis*, the causative agent of whooping cough (1). Using the expression construct pDO6935, previous studies have demonstrated *brkA* expression in *Escherichia coli* (1). However, the promoter driving *brkA* expression from this construct is unknown. pDO6935 harbors a *lac* regulatory region inclusive of a promoter, operator, and catabolite activator protein binding site (CAP BS) upstream of *brkA* which may be implicated in its expression. The lactose, or *lac*, operon regulatory elements are known for their ability to control gene expression via transcriptional regulation in *E. coli* (2). To characterize the *brkA* promoter on pDO6935 in *E. coli*, we created pLISA, a promoterless green fluorescent protein (GFP) vector to be used in a promoter trap experiment. In our work, regions upstream of the *brkA* locus on pDO6935, either inclusive or exclusive of the *lac* regulatory region, were cloned into pLISA. GFP expression was observed as an indicator of promoter activity. We showed that GFP expression was induced when amplicons contained the full *lac* regulatory region. This observation suggests that the *lac* regulatory region is likely both necessary and sufficient to drive *brkA* expression from pDO6935 in *E. coli* - informing future efforts to study virulence gene *brkA* using *E. coli* as a model system.

INTRODUCTION

Gene expression in *Escherichia coli* is driven by the interactions between promoters and RNA polymerases to initiate transcription, with transcription factors binding to promoters to result in increased or decreased regulation of transcriptional activity (3, 4). The *E. coli* genome encodes hundreds of transcription factors that bind promoters, recruit RNA polymerases, and drive gene expression (4, 5).

Arguably one of the most notable transcriptional regulatory systems, the lactose, or *lac*, operon was the first of its kind to be characterized (2). *E. coli* and many other bacterial species harbor this set of genes which encode proteins used for lactose metabolism (2). The expression of the *lac* operon is mediated by its interactions with the catabolite activator protein (CAP) and the repressor molecule LacI (2). LacI is constitutively expressed in *E. coli* and can form a homotetramer that binds DNA at the *lac* operator and prevents transcription (2). In a low-glucose environment, cyclic AMP (cAMP) production increases within the cell and combines with CAP to form a complex which binds the catabolite activator protein binding site (CAP BS) to induce expression (2). Further, when the extracellular environment lacks glucose and has high concentrations of lactose, a permease protein, LacY, transports lactose into the cell to be converted to allolactose (2). Allolactose then binds to LacI monomers to prevent tetramerization and subsequent suppression of the operon (2). Given that the *lac* operon is a modifiable gene expression system native to *E. coli*, this presents an

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opportunity to study the expression of genes from pathogenic bacteria using this model species.

Bordetella pertussis is a bacterial pathogen that is the causative agent for whooping cough; its genome contains a number of virulence genes which allow for the evasion of the immune system (1). The gene *Bordetella* resistance to killing A (*brkA*) encodes an autotransporter involved in adherence and serum resistance, through the blocking of the classical pathway of the complement cascade (6, 7). Many autotransporters, including BrkA, have been identified and remain a notable research topic due to the potential to be engineered as recombinant protein secretion systems (1). Previous research efforts have demonstrated BrkA expression in *E. coli* from plasmid constructs such as pDO6935 (1). In *B. pertussis*, *brkA* expression is driven by the *Bordetella* virulence gene (Bvg) system (1). However, the promoter controlling *brkA* expression from pDO6935 in *E. coli* remains unknown. The implications of finding this promoter include the ability to study virulence gene *brkA* in less stringent biosafety conditions and pose a potential method to regulate its expression. pDO6935 was cloned to contain the entire *brk* locus from *B. pertussis*, including both *brkA and brkB,* but excluding any 5' putative promoter sequence (1, 8).

pDO6935 harbors several key elements between its origin of replication (*ori*) and the 5' end of *brkA* (upstream of the translation start site (TSS)) that could be implicated in transcriptional regulation. These sites include the *lac* promoter, operator, and CAP BS. A previous study conducted in *E. coli* found BrkA to be constitutively expressed at low levels from pDO6935 (1). This observation was made without induction by the allolactose analog isopropyl-ß-D-thiogalactopyranoside (IPTG) and BrkA expression levels were deemed to be similar to those in *B. pertussis* (1, 7, 9). Here, the specific level of BrkA expression is significant because overexpression of the protein has been shown to be detrimental to bacterial cells (7). Thus, transcriptional regulation of *brkA* from pDO6935 occurs at a remarkably serendipitous level that allows for BrkA functionality and detection in *E. coli* without cell harm.

The regulatory elements of the *lac* operon are expressed in *E. coli* and would likely act on the *lac* promoter upstream of *brkA* on pDO6935. Thus, our study employs a promoter-trap method to determine whether the *lac* promoter is both necessary and sufficient to drive *brkA* expression in *E. coli*. We created a promoterless GFP vector, pLISA, into which we inserted different segments from pDO6935 – both inclusive and exclusive of the *lac* regulatory system – before evaluating GFP expression levels. Further, we stimulated the *lac* promoter with IPTG to induce GFP expression. Herein, we have described how fragments inclusive of the *lac* regulatory system (CAP BS, *lac* promoter, and operator) activated GFP expression from pLISA independent of IPTG induction. These results suggest that the *lac* regulatory elements are likely the sequences which drive *brkA* expression from pDO6935 in *E. coli*.

METHODS AND MATERIALS

Cultivation of bacteria and plasmids. pDO6935 (Oliver *et al*., 2003) and pSPPH21 (Gawol *et al.,* 2022) were acquired from the MICB 471 Lab (UBC) starter plate collection, as contained in *E. coli* DH5α. pSB1C3 was obtained from Dr. Avery Noonan (Hallam lab, UBC). All subsequent transformations were performed with NEB® 5-alpha Competent *E. coli* from the Q5 Site-Directed Mutagenesis kit (*New England Biolabs)* or competent DH5α cells (*Invitrogen*).

Primer design. Primers for polymerase chain reaction (PCR) and site-directed mutagenesis were created by selecting sequences of the template (pDO6935 or pSB1C3) on SnapGene software. Sequences and melting temperatures are indicated in Supplemental Tables S1 and S2. Primers were obtained from Integrated DNA Technologies (IDT). PUC19 primers were obtained from the MICB 471 Laboratory (UBC).

Luria Bertani (LB) media. LB media was created by dissolving tryptone, yeast extract, and sodium chloride (10 g, 5 g, and 10 g, respectively) in 1 L deionized water and separated into 250 mL batches for autoclaving (10). If LB-agar was desired, 3.75 g agar powder was added per 250 mL LB media before autoclaving (10). For media requiring antibiotics, ampicillin

(50 mg/mL in deionized water; 0.22 µm filter-sterilized) or chloramphenicol (CHL) (34 mg/mL in ethanol; 0.22 µm filter-sterilized) at a 1:1000 ratio (10).

Agarose gel electrophoresis. Agarose gels were produced using 1X TAE buffer to a concentration of 1% or 1.5% agarose depending on the application. RedSafe Nucleic acid stain (*FroggaBio*) was used in a 1:20,000 ratio of stain to gel. Samples were prepared by adding Gel loading Dye Blue 6x (*New England Biolabs*) in a 1:6 ratio of dye to sample. O'GeneRuler DNA Ladder Mix (*Thermo Scientific*) was loaded in the gel in 10 µL volumes. Agarose gels were run for 1 hour at 90 V followed by visualization with the ChemiDoc Imaging System (*Bio-Rad Laboratories*) on the SYBR Safe setting.

Overnight cultures. Cultures were grown in 4 mL of LB media in a shaken incubator at 37°C. If antibiotics were required, they were added to the media in 4 µL volumes.

PCR. According to the Invitrogen Platinum SuperFi DNA Polymerase User Guide, 50 µL PCR reactions were set up using the 5X SuperFi buffer (1X; *Invitrogen*), 10 mM dNTP mix (0.2 mM per dNTP; *Invitrogen*), 10mM forward primer (0.5 mM; *Invitrogen*), 10 mM reverse primer (0.5 mM; *Invitrogen*), 5X SuperFi Enhancer (1X; *Invitrogen*), and Platinum SuperFi II Polymerase (0.2 U/µL; *Invitrogen*) (11). Either 10 ng of pDO6935 template DNA or the equivalent volumes of water were added. PCR reactions were run in the Bio-Rad T100 Thermocycler at the temperatures suggested by the SuperFi Polymerase manufacturer (*Invitrogen*). Annealing temperatures were decided based on the melting temperatures of primers and were set to 3°C below the lowest temperature if primers had a melting point range within 5°C of each other. For reactions involving primers with greatly different annealing temperatures (>5°C), a touchdown PCR approach was used: the first three cycles were run at the highest melting temperature of the two primers, then the temperature was decreased each cycle by 1°C until a temperature 3°C below the lowest temperature primer was achieved (12). The remaining cycles were run at this temperature.

Plasmid extractions. Plasmid extractions were carried out using the EZ-10 Spin Column Plasmid DNA Miniprep Kit (*Bio Basic*) on 4 mL of overnight culture (https://www.biobasic.com/ez-10-spin-column-plasmid-dna-miniprep-kit-4155). The concentration and quality of extracted plasmids were determined using the Nanodrop Spectrophotometer (*Thermo Scientific*).

Cleanup of PCR products. PCR clean-up was performed using the GeneJET PCR Purification Kit (*Thermo Scientific*) as per the instructions provided by the manufacturer (https://www.thermofisher.com/order/catalog/product/K0701).

Restriction digests using XbaI and EcoRI-HF. All restriction digests were carried out at a volume of 50 µL per reaction with the following components: 10X rCutSmart Buffer (1X; *New England Biolabs*), EcoRI-HF (20 Units; *New England Biolabs*), XbaI (20 Units; *New England Biolabs*), 1 µg of template DNA, and DNase/RNase free water (https://nebcloner.neb.com/#!/protocol/re/double/EcoRI,XbaI). Reactions were carried out at 37°C for 2 hours in the Bio-Rad T100 Thermocycler. If the desired reaction was a single digestion, the volume of one enzyme was substituted with water. For uncut controls, the volume of both enzymes was substituted with water. If heat inactivations were performed, they were carried out at 65°C for 20 minutes in the Bio-Rad T100 Thermocycler.

Ligation. All ligation reactions were carried out at a volume of 20 µL per reaction with the following components: T3 DNA ligase buffer (2X), T3 ligase (1 µL), and water (https://nebcloner.neb.com/#!/protocol/Ligation/M0317). DNA was added in a ratio of 1:3 of vector to insert, respectively (as calculated using the *NEBiocalculator*) (https://nebiocalculator.neb.com/#!/ligation). Reactions were carried out at 25°C for 15 minutes in the Bio-Rad T100 Thermocycler.

Transformation. Competent DH5α cells (*Invitrogen*) were transformed with 5 µL of ligated plasmid DNA (pEAH23A-pEAH23E) produced by the ligation reactions. After plasmid addition to 50 µL DH5α cells, they were incubated on ice for 30 minutes, heat-shocked in a 42°C water bath for 30 seconds, and incubated on ice again for 5 minutes. 950 µL SOC Medium or LB was added to each tube, and cells were incubated at 37°C in the shaking incubator. Finally, 75 µL of the transformed cells were spread onto LB-CHL plates and incubated overnight at 37°C (http://cmdr.ubc.ca/bobh/method/cacl2-transformation -of-e-coli/, 13, 14).

Q5 site-directed mutagenesis. According to the New England Biolabs Q5 Site-Directed Mutagenesis Protocol, a 25 µL reaction was set up with the Q5 Hot Start High-Fidelity 2X Master Mix (1X; *New England Biolabs*), 10 µM Forward Primer (0.5 µM; *New England Biolab*s), 10 µM Reverse Primer (0.5 µM; *New England Biolabs*), template DNA (~17 ng/µL pSB1C3), and nuclease-free water (*Invitrogen*). The reaction was run in the Bio-Rad T100 Thermocycler according to the New England Biolabs protocol and used an annealing temperature of 57°C. Finally, the subsequent KLD reaction and transformation were carried out according to the manufacturer's instructions and incubated at 37°C overnight (https://www.neb.com/en/protocols/2013/01/26/q5-site- directed-mutagenesis-kit-protocol-e0554). The next day, the plates were screened in the ChemiDoc Imaging System (*Bio-Rad Laboratories*) set to Alexa546 and colonies that were negative for GFP expression were selected before proceeding.

IPTG plate induction and fluorescence visualization. For conducting IPTG plate induction, 10 µL of 1 M IPTG was spread on the surface of cooled LB-CHL plates which were subsequently inoculated with transformant clones (A-E). Each clone was also inoculated in LB-CHL plates without IPTG. All plates were incubated at 37ºC overnight. The following day, the plates were imaged using the ChemiDoc Imaging System (*Bio-Rad Laboratories*) set to Alexa546 to observe GFP expression (15).

IPTG broth induction and GFP fluorescence quantification. For conducting IPTG broth induction, 200 µL reactions were set up in a black-walled 96-well plate with a clear bottom. IPTG was added to a final concentration of 0.5 mM in LB-CHL or deionized water was added for uninduced reactions. Individual colonies from pEAH23A-E, pDO6935, pSPPH21, or pSB1C3 were inoculated into each well, and the plate was incubated overnight in a microplate reader (*BioTek*) at 37°C while shaking. Before taking a measurement, wells were manually mixed with a pipet. The plate was measured for optical density (OD_{600}) and GFP (excitation 485 nm, emission 528 nm) in the microplate reader (*BioTek*) (15).

Statistical analysis of fluorescence quantification. GFP expression in each well of the microplate was normalized to optical density (OD_{600}) by dividing the GFP signal by the OD_{600} measurement. Next, the normalized GFP expression level of each well was further normalized to the vector control which received the same treatment (IPTG or water) to show relative GFP expression. Statistical analysis (2-way ANOVA test) was performed in GraphPad Prism™.

Whole plasmid sequencing. Plasmid concentrations and purity were evaluated using the Nanodrop apparatus (*Thermo Scientific*). If necessary, dilutions were performed to fit sequencing specifications before being sent to Plasmidasaurus for whole-plasmid nanopore sequencing. Results were analyzed using SnapGene.

RESULTS

Creation of promoterless GFP vector (pLISA) via site-directed mutagenesis of pSB1C3-GFP promoter. To establish a suitable GFP-reporter system for evaluating gene expression, we removed the promoter from pSB1C3, a GFP expression vector for *E. coli*. Initial screening of pSB1C3 for promoter activity revealed GFP expression at all volumes of 1 M IPTG added (0 µL, 10 µL, 50 µL) (Supplemental Fig. S1A+S1C). This indicated that the GFP expression was not IPTG-inducible in pSB1C3. In contrast, the control vector pSPPH21 (plasmid expressing GFP under the control of a *lac* regulatory region) displayed increasing

GFP expression levels in the same experimental setup, validating IPTG induction as a testing method (Supplemental Fig. S1C).

Further investigation identified the BBa_J123110 promoter upstream of the GFP locus in pSB1C3 (http://parts.igem.org/Part:BBa_J23100). Using the SnapGene software, mutagenesis primers were designed to truncate most of BBa_J123110 (Supplemental Table S1). Following cloning, *E. coli* colonies on transformed plates were screened for GFP activity in the ChemiDoc Imaging System (Alexa546; *Bio-Rad Laboratories*). As depicted in Fig. 1A (III), the transformed plate displayed both GFP positive and negative colonies: appearing similar to the positive and negative controls (Fig. 1A (I) and Fig. 1A (II), respectively). To

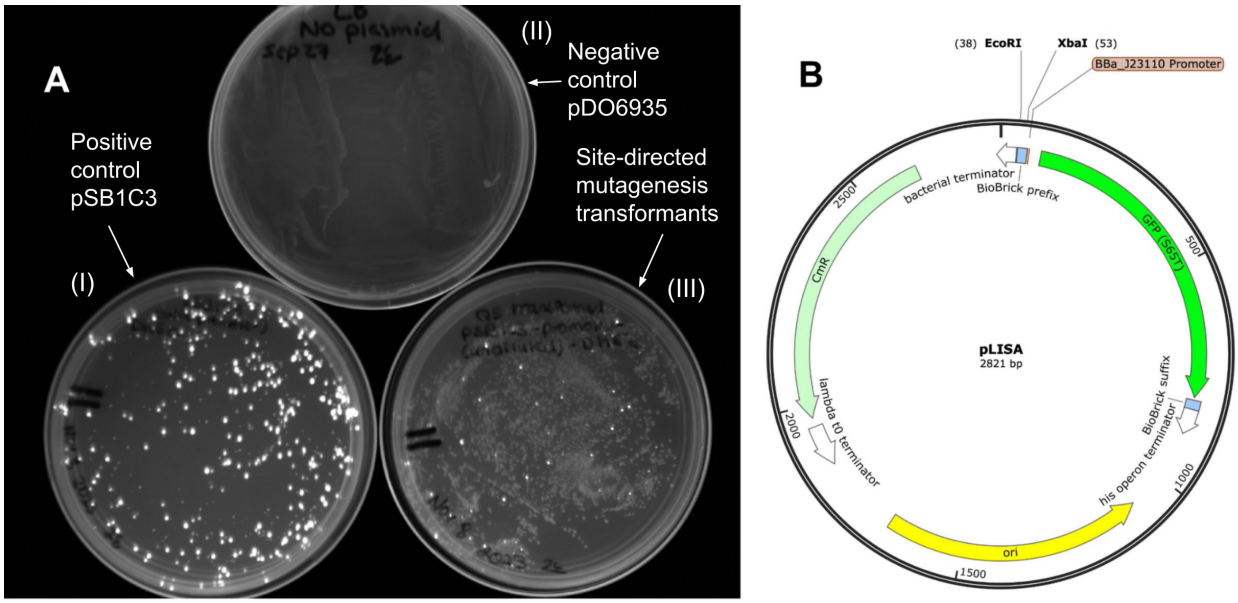

FIG. 1 Successful creation of promoterless-GFP plasmid: pLISA. (**A**) Visualization of GFP expression using the ChemiDoc Imaging Apparatus (Bio-Rad Laboratories) at Alexa546. (Top plate) Negative control of untransformed *E.coli* DH5α cells. (Bottom Left Plate) Positive control of cells containing pSB1C3. (Bottom Right Plate) Competent NEB® 5-alpha *E. coli* transformed with resultant plasmids from site-directed mutagenesis. (**B**) Map of pLISA, the resultant promoterless GFP vector from site-directed mutagenesis. Key genetic elements are annotated.

protect the plasmid DNA integrity, 12 colonies on the other transformant plate (not screened under UV) were selected for further screening. These 12 colonies were inoculated into LB with chloramphenicol (LB-CHL) broths and plates and grown overnight at 37°C. Plates were screened the following morning for GFP expression and we observed that only colony 5 was found to be GFP positive (Supplemental Fig. S1D). Plasmid extractions were performed on the overnight cultures of all other colonies. Colonies 6, 7, and 9 were chosen to proceed based on observed GFP expression level. Next, plasmids were screened for size and inclusion of XbaI and EcoRI-HF restriction endonuclease sites via digestion with both enzymes and gel electrophoresis on a 1% agarose gel (Supplemental Fig. S1E). We observed that all three plasmids were the correct size (~3000 bp) and included both cut sites. Based on purity and yield, vector 6 was selected for further experimental use as a novel promoterless GFP vector: pLISA.

Five regions upstream of *brkA* on pDO6935 were amplified via PCR. To investigate whether the *lac* promoter is responsible for driving *brkA* expression, five distinct nucleotide regions upstream of the *brkA* gene locus were amplified from pDO6935 via PCR (Fig. 2A + 2B, Supplemental Table S4). These regions were selected to either include or exclude the *lac* regulatory elements (Fig. 2B). The amplification process employed primers which incorporated restriction endonuclease sites, either XbaI or EcoRI-HF, within a 5' overhang to facilitate subsequent ligation into pLISA (Supplemental Table S2). PCR products were confirmed via gel electrophoresis (Fig. 2C). We observed that all PCR products were visible at their expected sizes (Supplemental Table S3). We also observed faint bands in the no template control lanes 4 and 10, which are possibly due to spillover when samples were

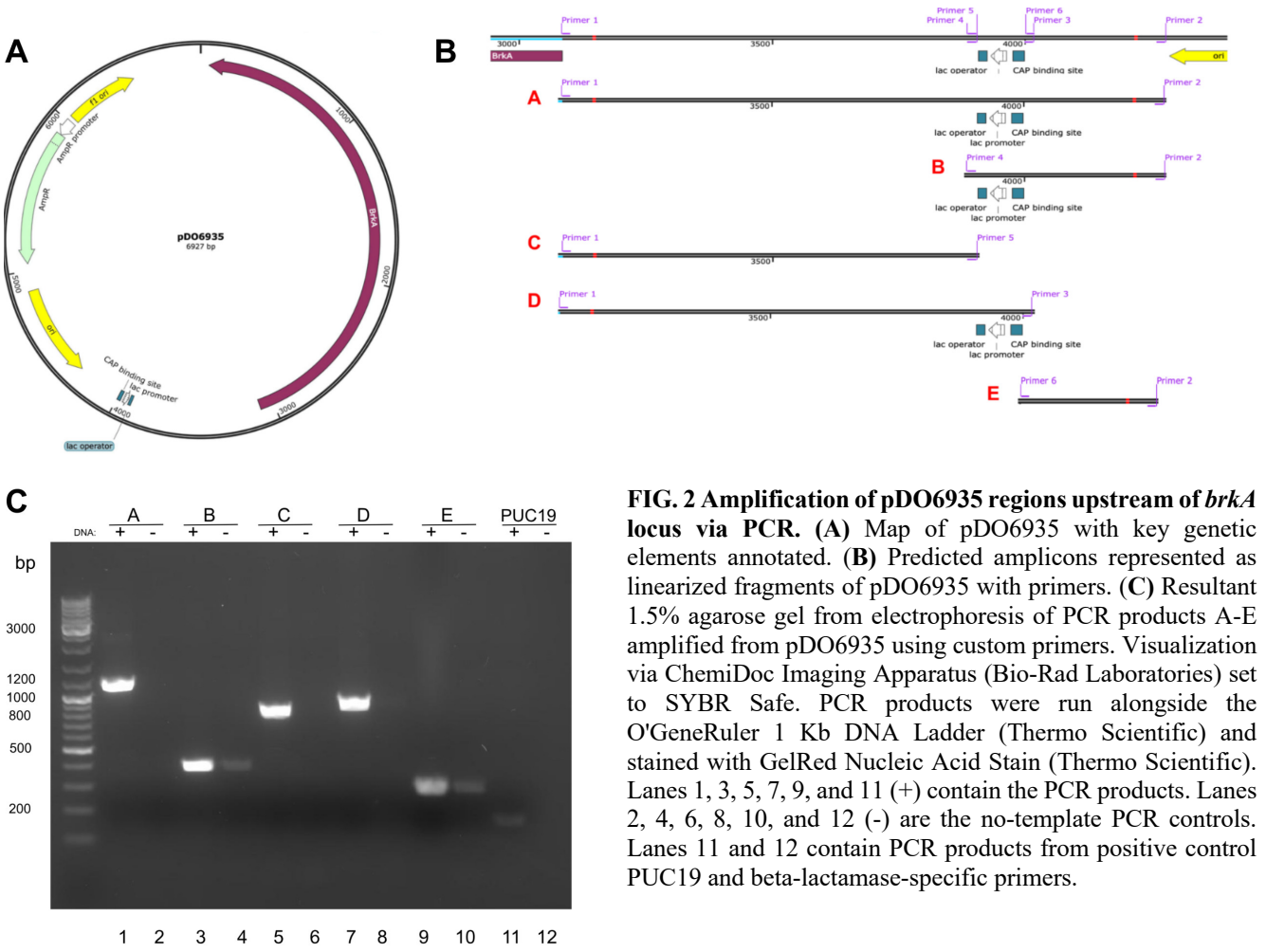

FIG. 2 Amplification of pDO6935 regions upstream of *brkA* locus via PCR. (A) Map of pDO6935 with key genetic elements annotated. **(B)** Predicted amplicons represented as linearized fragments of pDO6935 with primers. **(C)** Resultant 1.5% agarose gel from electrophoresis of PCR products A-E amplified from pDO6935 using custom primers. Visualization via ChemiDoc Imaging Apparatus (Bio-Rad Laboratories) set to SYBR Safe. PCR products were run alongside the O'GeneRuler 1 Kb DNA Ladder (Thermo Scientific) and stained with GelRed Nucleic Acid Stain (Thermo Scientific). Lanes 1, 3, 5, 7, 9, and 11 (+) contain the PCR products. Lanes 2, 4, 6, 8, 10, and 12 (-) are the no-template PCR controls. Lanes 11 and 12 contain PCR products from positive control PUC19 and beta-lactamase-specific primers.

loaded into the gel. Overall, all five regions were successfully amplified via PCR and ready for digestion and insertion into pLISA.

Five amplicons were ligated into pLISA and clones were transformed into *E. coli* DH5α cells. To create complementary ends to allow for amplicon ligation into pLISA, a double restriction digest of five amplicons (A-E) and pLISA was conducted, followed by ligation into the pLISA vector. As clones A, B, and D were expected to activate GFP expression, they were screened for GFP expression under short-wavelength UV light. Five colonies per clone were selected and inoculated in LB broth and agar plates for overnight growth and plates were screened using the ChemiDoc Imaging System. Following selection and transformation, we observed dim GFP expression from all Clone A colonies, bright GFP expression from 5 of 6 Clone B colonies, and no GFP expression from any Clone D colonies (Supplemental Fig. S2A + S2B).

To screen Clones C and E, which were expected to be GFP negative (given that they do not include the *lac* regulatory regions), a double restriction digest (using EcoRI-HF and XbaI) and gel electrophoresis were performed. We observed that all five colonies from Clone C and all 4 colonies from Clone E included inserts at the expected sizes of ~839 bp and ~295 bp, respectively (Supplemental Table S3, Supplemental Fig. S2C). Plasmid extractions were performed on colonies A5, B5, and D5 and digested with EcoRI-HF and XbaI, then run on a 1% agarose gel alongside colonies C5 and E2 (Fig. 3). We observed that all 5 clones displayed the expected bands: one band of the cut vector at ~3000 bp and one band at the correct insert size, although the appearance of E2 is faint (Supplemental Table S3). We also observed some bands above the ~3000 bp cut vector, which roughly correlate to the size of an undigested vector and insert. As Clones A5, B5, C5, D5, and E2 all contained the expected insert sizes, the ligation of amplicons A-E into pLISA was confirmed and these clones were chosen to proceed in further experimentation.

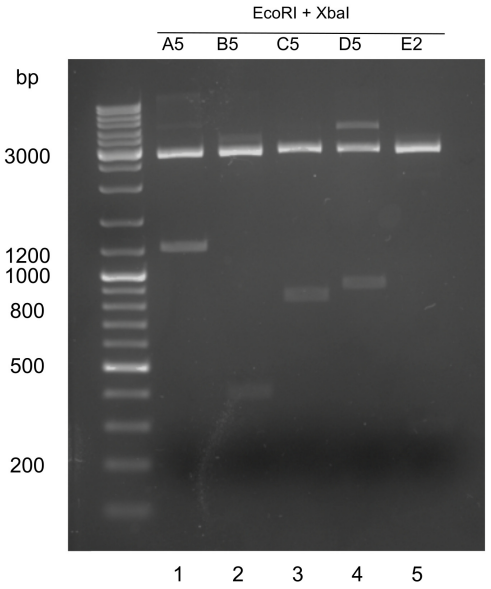

FIG. 3 Restriction digests confirm the proper insertion of amplicons A-E into pLISA. Ligated plasmids (A-E) were extracted from transformed *E. coli* DH5α and subjected to restriction digestion by EcoRI and XbaI. The resultant products were run on a 1.5% agarose gel using GelRed Nucleic Acid Stain (Thermo Scientific) and visualized in the ChemiDoc Imaging Apparatus (BioRad-Laboratories) at Alexa546. Lanes 1-5 contain the digested Clones A-E respectively, with bands visible for both the digested plasmids and inserts (A-E). Digestion products were run alongside the O'GeneRuler 1 Kb DNA Ladder (Thermo Scientific).

Two clones containing the *lac* regulatory region activated GFP expression independent of IPTG induction. To observe if induction of the *lac* regulatory system would increase GFP expression, Clones A-E were grown in the presence or absence of IPTG. A qualitative assay was performed, in which 10 µL of 1 M IPTG was spread onto LB-CHL plates. Clones A-E were grown overnight at 37°C and subsequently imaged in the ChemiDoc Imaging System. When all five clones were streaked onto a single plate, we observed that only Clone B was visibly GFP positive and displayed similar expression to the positive control, pSB1C3 (Fig. 4A + 4B). Further, we observed that GFP expression was independent of IPTG induction (Fig. 4A + 4B). When plates streaked with only single clones were imaged, we observed that Clone A also appeared equally GFP positive in the presence and absence of IPTG (Supplemental Fig. S3A). Finally, we observed that Clones C, D, and E did not express GFP: appearing similar to our negative control, pLISA (Supplemental Fig. S3C, D, E). Therefore, it was observed that only Clones A and B (both including the *lac* regulatory region) could activate GFP production and IPTG induction did not affect GFP expression.

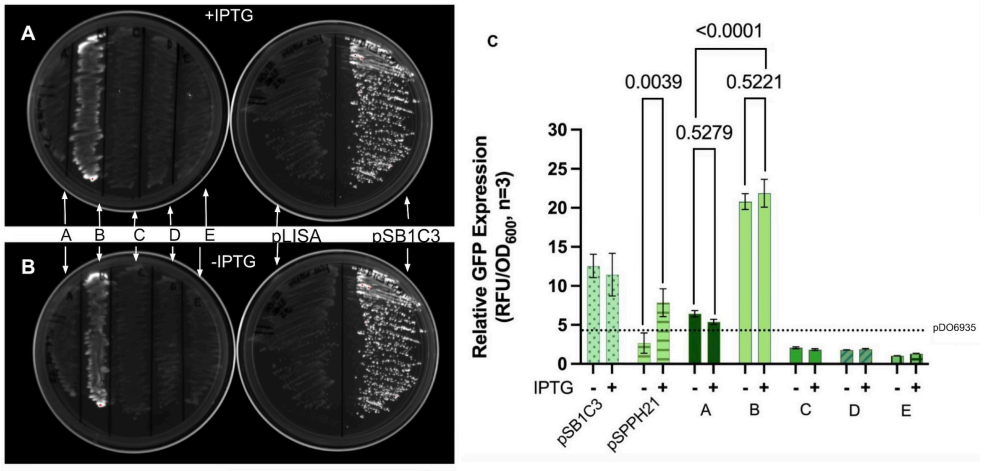

FIG. 4 Clones A and B drive GFP expression regardless of IPTG induction. (A and **B)** Visualization of GFP expression from *E. coli* DH5α cells transformed with pEAH23A-pEAH23E induced with 10 µL 1 M IPTG by spread-plating on LB-CHL plates (top left) or without IPTG on LB-CHL plates (bottom left). GFP-expression controls pLISA (-) and pSB1C3 (+) induced with 10 µL 1M IPTG by spread-plating on LB-CHL plates (top and bottom right). Images were captured using the ChemiDoc Imaging Apparatus (BioRad-Laboratories) at Alexa546. **(C)** Results of IPTG induction in liquid culture as measured by the microplate reader (BioTek). Colonies containing pEAH23A-pEAH23E, pDO6935, pSPPH21, or pLISA were grown at 37°C overnight in a 96-well plate in triplicate with 0.5 mM IPTG or water. GFP measurements in each well are normalized to optical density and standardized to pLISA. The dotted black line is the normalized negative control (pDO6935) relative to pLISA. Results were plotted in GraphPad Prism using SEM and statistical analysis was conducted via a 2-way ANOVA test ($p<0.05$). p values are plotted above the graph to indicate the significance between treatment groups.

A quantitative IPTG induction assay was set up to measure optical density (OD_{600}) and GFP signal. To complete the analysis, each well's fluorescence reading was normalized to optical density. Next, Clones (A-E), a constitutive positive control (pSB1C3), an IPTG-inducible positive control (pSPPH21), and a negative control (pDO6935) were normalized to pLISA using pairwise analysis (Fig. 4C). pDO6935 possessed a higher GFP expression value than expected (~4.3 RFU/OD_{600}, n=3) (Fig. 4C). This was attributed to its poor growth causing a lower optical density and thus a slight skewing of GFP expression when values were normalized. Clones C, D, and E were reported to be GFP negative, with RFU/OD_{600} values of ~1.9, ~1.8, and ~1.1, respectively (Fig. 4C). Further, Clones A and B were reported to be GFP positive, with RFU/OD_{600} values of ~5.9 and 21, respectively (Fig. 4C). These later clones have higher relative RFU/OD_{600} values than those observed in pDO6935. There was no significant increase in GFP expression when IPTG was added to Clones A or B ($p = 0.5279$ and 0.5221, respectively) (Fig. 4C). pSPPH21 demonstrated a significant increase in GFP expression ($p = 0.0039$); thus, validating the IPTG induction assay (Fig. 4C). Notably, Clone B expressed significantly more GFP than Clone A ($p < 0.001$): supporting similar observations from the qualitative IPTG induction (Fig. 4A, B, C). Overall, these results suggested that GFP expression from Clones A and B was independent of IPTG induction.

pLISA and pEAH23A, pEAH23B, pEAH23C, pEAH23D, and pEAH23E sequences confirmed via whole plasmid sequencing. To confirm the sequence of Clones A, B, C, D, E (renamed to pEAH23A, pEAH23B, pEAH23C, pEAH23D, pEAH23E, respectively), and pLISA, we sent plasmids for nanopore sequencing via Plasmidsaurus. After sequence analysis using SnapGene, we found pLISA to align with the expected sequence from site-directed mutagenesis. pLISA contained the successful knockout of the BBa_J123110 promoter while maintaining the integrity of the restriction endonuclease sites for XbaI and EcoRI-HF (Supplemental Fig. S4A-E). Additionally, these results were confirmed to be of high quality, with a sequence coverage of ~1220X. Analyses of pEAH23A, pEAH23B, pEAH23C, and pEAH23E displayed that the regions inserted upstream of the GFP locus were homologous to the expected sequences and at the correct sizes (see Fig. 2B). Sequence coverages for these reads were 942X, 2520X, 1900X, and 2470X, respectively. The sequencing results for pEAH23D showed that the construct did not include the CAP BS at the upstream end of the *lac* regulatory region as it was expected to and had a sequence coverage of 251X (Supplemental Fig. S4D). These results showed that each sequence possessed a highly accurate consensus, with all constructs, except pEAH23D, containing the hypothesized sequences.

DISCUSSION

In this study, we conducted site-directed mutagenesis to create the promoterless GFP vector, pLISA, which we used to determine the *brkA* transcriptional regulatory system in *E. coli* DH5α. By cloning different segments from pDO6935 upstream of the GFP on pLISA, we showed that all segments inclusive of the *lac* promoter, operator, and CAP BS (Clones A and B) were able to activate GFP expression. These results suggested that this regulatory region may drive *brkA* transcription from pDO6935 in *E. coli* DH5α. We initially hypothesized that Clone D would also activate GFP expression, as it was predicted to be inclusive of the entire *lac* regulatory region. However, it was found that Clone D did not express GFP. Through analysis of Clone D sequencing results, it was revealed that the CAP BS was truncated during PCR amplification of the segment. Not only did this observation explain the lack of fluorescence produced by Clone D, but further supported the hypothesis that the *lac* regulatory region is responsible for *brkA* transcription. Since the CAP BS is an essential component of the *lac* regulatory element, truncation of this region would likely abolish GFP expression (16).

Next, we sought to test whether the introduction of IPTG would induce higher levels of GFP expression in Clones A-E. By performing IPTG induction assays in solid and liquid media, we were able to examine GFP expression levels qualitatively and quantitatively through comparison with uninduced cells. Between Clones A and B, there is no apparent increase in GFP expression when grown on solid media containing IPTG. These results are supported by liquid culture induction read by a microplate reader which did not show a significant increase in GFP expression between induced and uninduced samples. Differently,

our IPTG induction control (pSPPH21 from *Gawol et al.,* 2022) showed a significant increase in GFP expression in response to IPTG induction: affirming that Clones A-E were not induced in this manner. One possible explanation for this observation is the level of LacI in *E. coli* DH5α cells, from which Clones A-E are derived. LacI acts as a repressor of the *lac* promoter by binding the operator region and blocking transcription (2). When lactose, or an allolactose-analogue (IPTG), is introduced, it binds LacI to prevent *lac* promoter repression (2). Upon further research, we found that while the *E. coli* DH5α strain expresses LacI, the copy number of the gene is too low to sufficiently suppress *lac* promoter activity (17). Therefore, we can surmise that GFP expression in Clones A and B was not significantly increased by IPTG induction because of the insufficient initial suppression of the *lac* promoter. Thus, the purpose of IPTG induction, to increase promoter activity, was ineffective.

Overall, we induced GFP expression by cloning the predicted promoter region of *brkA* (the *lac* regulatory region) from pDO6935 into pLISA. These results indicated that the *lac* promoter is likely both necessary and sufficient to drive *brkA* expression in *E. coli* transformed with pDO6935. We inferred the necessity of the *lac* promoter from the results of Clones C and E, which do not include the *lac* promoter. Given that GFP expression was abolished in these clones, it is suggested that the *lac* promoter is likely an essential element in expression. Likewise, the sufficiency of the *lac* promoter can be inferred through comparing Clones B and E, which possess identical regions that differ by *lac* promoter inclusion. Clone B includes the *lac* regulatory elements while Clone E does not. As Clone B was able to express GFP while E could not, it is likely that the differential region between both clones (the *lac* regulatory elements) is sufficient in driving GFP expression. Interestingly, there was a significant increase in GFP expression between Clones A and B. While both clones contain the same elements of the *lac* regulatory system, they differ in proximity to the GFP locus (Supplemental Fig. S4A + S4B). Thus, further research into the relationship between *lac* promoter proximity to a gene could aid in explaining this phenomenon. Given that we observed the *lac* promoter to be both necessary and sufficient to drive gene expression of GFP on pLISA, our hypothesis that the *lac* promoter is driving the expression of *brkA* on pDO6935 is supported. Since *brkA* can be expressed and regulated in *E. coli*, this may constitute an easier and safer method of studying this *B. pertussis* virulence gene. Given that our results suggest *brkA* is under the control of the *lac* regulatory system, IPTG induction or glucose-based repression can both be used to finely modulate *brkA* expression levels.

Conclusions In this study, we constructed pLISA as a promoterless GFP vector to be used in cloning experiments using *E. coli* DH5α. We illustrated that the insertion of regions from pDO6935 containing the complete *lac* regulatory system activated GFP expression from pLISA. Likewise, regions which excluded the *lac* regulatory system or possessed a *lac* system with a truncated CAP BS were unable to drive GFP expression. Thus, we have demonstrated that the *lac* regulatory system is likely both sufficient and necessary to drive GFP expression from pLISA. Therefore, the *lac* regulatory system may be the promoter responsible for controlling *brkA* transcription from pDO6935 in *E. coli*. Finally, we also showed that in *E. coli* DH5α, *lac* induction of pLISA by IPTG was ineffective and likely requires a different model system for future experiments.

Future Directions To further support the results of our experiments, future projects could utilize site-directed mutagenesis to remove or disrupt the *lac* regulatory system on pDO6935 to confirm that *brkA* transcription is abolished. Additionally, given that Clone D (pEAH23D) possessed a truncated CAP BS, future experimentation could design new primers and repeat our experimental steps to confirm that the region between *brkA* and the 5’ end of the *lac* regulatory system can indeed drive GFP expression. We have demonstrated the necessity and sufficiency of the *lac* regulatory system for *brkA* transcription from pDO6935. Future experiments could use this information to replace the *brkA* locus with other genes and utilize pDO6935 as a *lac*-inducible system with IPTG. Finally, pLISA has applications in a wide variety of molecular cloning experiments. Our novel promoterless GFP vector could be used for other promoter trapping experiments or for comparisons of promoter intensity.

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The contents of this manuscript were written and revised as a collaborative effort between E.H., A.T., and H.A. The project was originally conceived by E.H. and the concept was further refined equally by all three members. Laboratory efforts were shared amongst all members.

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