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The *lac* Promoter May Not Regulate *brkA* Expression in Plasmid pDO6935 in *Escherichia coli*

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SUMMARY BrkA is an autotransporter protein found in *Bordetella pertussis* that is regulated by the Bordetella virulence gene (Bvg) system. It has been researched in pDO6935-transformed *Escherichia coli*, with previous research suggesting that the presence of a lac promoter is necessary for BrkA expression in these models. *lacI*^{Q} is a constitutively expressed gene found in pHN678 that encodes the LacI repressor, which when in the absence of lactose or analogs, should bind to the operator downstream of the lac promoter, thereby preventing downstream gene expression. Therefore, the objective of this study was to manipulate this mechanism by introducing *lacI*^{Q} into a pDO6935-derivative, pENS, to create an IPTG-inducible BrkA expression system in *E. coli* cells, assuming that the lac promoter was responsible for BrkA expression. We created pTEAM, a construct containing *lacI*^{Q} downstream of BrkA on the pDO6935-derived pENS plasmid, which was transformed into UT5600 *E. coli* cells prior to visualization for BrkA expression. Contrary to our hypothesis, our data showed that BrkA was still being expressed with strong signals by our pTEAM construct in the absence of IPTG whereas our pENS constructs expressed little to no BrkA. This result suggests that the lac promoter may not regulate BrkA expression in pDO6935.

INTRODUCTION

BrkA is an autotransporter protein and virulence factor found in *Bordetella pertussis*, a gram-negative bacterium responsible for causing the contagious illness whooping cough (1, 2). Studies have shown that *Escherichia coli* models transformed with plasmid pDO6935 will express BrkA on the cell surface, allowing for a non-pathogenic model for BrkA research (3). However, while *brkA* expression in *B. Pertussis* has well-characterized regulation under the Bordetella virulence gene (Bvg) system (1), the regulatory system responsible for *brkA* expression in *E. coli* models transformed with pDO6935 is poorly characterized.

The *lac* operon is a transcriptional regulatory system essential for lactose metabolism in bacteria such as *E. coli*. It encodes for one transcriptional product containing three proteins (LacZ, LacY, and LacA) (4, 5). The regulation of the *lac* operon involves a promoter that is controlled by the concentration of lactose in the environment and the homotetramer repressor LacI; LacY transports lactose into the cell, LacZ catalyzes lactose isomerization to allolactose, and allolactose binds to LacI monomers to prevent its tetramerization (4, 5). In a lactose-free environment, the LacI repressor binds the operator site and prevents RNA polymerase from binding; otherwise, lactose binds to the LacI repressor, freeing it from the promoter to allow transcription of the *lac* operon.

A recent study by Haniak et al. suggested that the *lac* regulatory system may contain the promoter responsible for *brkA* expression in *E. coli* transformed with pDO6935, providing strong evidence that a complete *lac* regulatory system was sufficient and necessary for activated GFP expression in promoterless vectors (6). In an isolated model, we expect to see no BrkA expression from pDO6935-*E. coli* in the absence of allolactose, as LacI would physically bind

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and block *lac* operon transcription. However, BrkA is constitutively expressed in these models even in the absence of allolactose analog, IPTG (Isopropyl ß-D-1-thiogalactopyranoside). This was theorized to be because pDO6935 and its 6XHis-tagged BrkA derivative, pENS, have high-copy numbers, with approximately 300-500 copies per cell (7), and do not include a LacI repressor gene. This could result in insufficient genomic LacI expression to regulate the high copy plasmids, leading to a lack of allolactose-dependent control over *lac* operon transcription and subsequent BrkA expression (8).

Aiming to improve regulatory control over the *lac* promoter and subsequent BrkA expression by addressing the issues that were theorized to be occurring in pENS, we intend to introduce a constitutively-expressed LacI sequence (*lacI*^{Q}) into the plasmid upstream of the *lac* operon. The decision to use *lacI*^{Q} was based on two factors. First, *lacI*^{Q} has a CG-to-TA change located in the -35 region of the LacI promoter, resulting in a 10-fold increase in expression of the LacI repressor (9). Thus, the high expression of LacI can block unwanted *lac* promoter activity even within high-copy-number plasmids such as pENS. Second, LacI's repressor activity can be prevented through induction with IPTG, activating *lac* promoter transcription easily using an inducible model (10). This induction mechanism using IPTG would also retain the perks of using an inducible system for protein expression including the ability to run time-dependent expression studies and a reduction of protein toxicity from extended expression (11).

Our new plasmid system has been named pTEAM. We expect that the insertion of *lacI*^{Q} into pENS would increase LacI expression at a quantity sufficient to repress the *lac* operons on the high-copy pENS plasmids controlling BrkA expression. As such, pTEAM would have tight IPTG-modulated regulation of BrkA expression, assuming that BrkA expression is controlled by the lac operon.

METHODS AND MATERIALS

Primer Design. The primers shown in Table 1 were designed to bind the plasmid pHN678 for the creation of a *lacI*^{Q} DNA fragment with added homologous extensions matching the regions flanking the HindIII cut site in pENS. The primers were each designed in two parts, the first being the homologous extensions matching pENS and the second being the sequence containing *lacI*^{Q} in pHN678. The homologous extensions were designed to include the 19 bases either upstream or downstream of the forward and reverse primers respectively (allowing for directionality of insertion) along with the "AAGCT" sequence comprising each end of the HindIII-digested pENS plasmid. Twenty more bases were added to match sequences on pHN678 upstream of the *lacI*^{Q} promoter site for the forward primer and downstream of the end of the protein for the reverse primer. The exact location of the matching sequences was decided upon while taking care to ensure that the HindIII site was not re-created, and that the melting temperature for each primer was as close to each other as possible. T_m for the forward primer is 53°C on pHN678 (20 bases bound), and 70°C with all 44 bases bound, while the T_m for the reverse primer is 60°C on pHN678 and 74°C with all 44 bases bound.

TABLE. 1 Primers used for the extraction of the *lacI*^{Q} gene from pHN678.

Primer Name	Sequence (5' - 3')	Annealing Temperature (°C)
lacIq_forward	TCTGCAGGAATTCGATATCAAGCTCGTTGACACCATCGAATGGT	70
lacIq_reverse	CGAGGTCGACGGTATCGATAAGCTGCTCACTGCCCGCTTTCCAG	74

***lacI*^{Q} PCR.** 50µL PCR reactions were set up using 5µL 10x Taq DNA Polymerase PCR Buffer (Invitrogen), 2.5µL 10mM dNTP mix (Invitrogen), 5µL 25mM $MgCl_2$, 3.5µL DMSO, 2.5µL 10µM forward primer, 2.5µL 10µM reverse primer, 30ng pHN678, and 1µL Taq Polymerase (Invitrogen). Bio-Rad T100 thermocyclers were used to run PCR under the following conditions: Initial denaturation at 95°C for 3 minutes; Denaturation at 95°C for 30 seconds over 33 cycles, either: 1. Initial forward primer annealing at 55°C for 30 seconds over 4 cycles, 2. Initial reverse primer annealing at 48°C for 30 seconds over 4 cycles, 3. Full primer annealing at 64°C for 30 seconds over 25 cycles, Extension at 68°C for 1 minute over 33 cycles; Final extension at 68°C for 5 minutes.

pENS Digest. 50µL digest reactions were set up using 5µL 10x rCutSmart buffer (NEB), 20 units of HindIII-HF (NEB), and 1µg of pENS isolate. Bio-Rad T100 thermocyclers were used to run the digest at 37°C for 3 hours.

Clean Up of PCR Products and Digest. The BioBasic EZ-10 Spin Column PCR Products Purification Kit was used to clean up the *lacI*^{Q} PCR and pENS digest. 30µL of *lacI*^{Q} PCR mixture, or 20µL of pENS digest, was incubated for 2 minutes in 5 volumes of Buffer B3, added to the EZ-10 flow through columns, and centrifuged (10,000 rpm, 2 minutes). Flow through was discarded. Columns were then washed twice with 750µL of Wash Solution, centrifuging (10,000 rpm for 2 minutes) and discarding flow through. DNA was then eluted in 30µL or 6µL of Elution Buffer, respectively, incubating for 2 minutes before centrifuging (10,000 rpm, 2 minutes). Nanodrop was used to determine sample concentration.

Gibson Assembly. A 10µL Gibson Assembly reaction was set up using 85.55ng linearized pENS (to 0.04 pmol), 14.96ng *lacI*^{Q} insert (to 0.04 pmol), and 5µL GeneArt Gibson Assembly EX Master Mix A (Thermofischer). A Bio-Rad T100 Thermocycler was used to incubate the mixture with the following specifications: 37°C for 5 minutes; 75°C for 20 minutes; 60°C for 30 minutes, cooling by 0.1°C/second; 4°C hold, cooling by 0.1°C/second. 10µL of GeneArt Gibson Assembly EX Master Mix B (Thermofischer) was then added to the reaction mixture and incubated at 45°C for 15 minutes. A fraction of the final Gibson Assembly product was then re-digested with HindIII-HF to linearize suggested residual, undigested pENS plasmid; a 50uL reaction was set up using 5µL 10x rCutSmart buffer (NEB), 20 units of HindIII-HF (NEB), and 5µL Gibson Assembly product. Bio-Rad T100 thermocyclers were used to run the digest at 37°C for 3.5 hours.

Agarose Gels. 100mL agarose gels of 1.5% (PCR), 1% (plasmid isolation), and 0.8% (plasmid isolation) were made using 100mL 1x TAE, 5uL RedSafe (FroggaBio), and respective agarose quantities. A range of 100-200 ng of O'GeneRuler DNA Ladder Mix (Thermo Scientific), 100-200 ng of plasmid isolates, and 5-10µL of PCR reaction mix were loaded across different gels with respective 6x DNA loading dye (Fermentas) volumes. Gels were run in 1x TAE buffer at 100V until loading dye reached ⅓ down the gel (1.5 hours) and images were obtained using a BioRad ChemiDoc MP Imaging System.

Competency. In accordance with by the CaCl2 Transformation of *E. coli* Protocol from the Hancock Lab, 100 mL of sterile LB was inoculated with 1mL of UT5600 *E. coli* overnight culture and grown at 37°C (with shaking) until an OD550 between 0.2 and 0.4 was met (~2 hours) (12). Cells were then kept on ice for 10 minutes and transferred to 2 Oakridge tubes. Cells were then collected as pellets by centrifugation at 10,000 rpm for 5 minutes at 4C, resuspended in 50mL 0.1M $CaCl_2$, and kept on ice for 20-40 minutes. Cells were collected as pellets by centrifugation at 10,000 rpm at 4°C for 5 minutes and resuspended in 4mL of 0.1M $CaCl_2$ and either immediately transformed or stored in 15% glycerol at -70°C.

Transformation. For transformation of the UT5600 strain of *E. coli* with pENS isolate (1), 25 ng of pENS was added to 1mL of competent cells. For the transformation of One Shot TOP10 Chemically Competent *E. coli* (Thermofischer) with pTEAM from Gibson Assembly (2), 1µL of 6x diluted Gibson Assembly mix, or 1µL of Gibson Assembly HindIII-HF digest, was added to 50µL of competent cells. For the transformation of UT5600 *E. coli* with pTEAM isolate (3), 150 ng of pTEAM was added to 50µL of competent cells. Cells were kept on ice for 10-30 minutes, heat shocked for 30-60 seconds, and placed on ice for at least 2 minutes. Cells were then added to 1mL LB (1) or 500µL SOC (2, 3) and incubated at 37°C for 1 hour. 100µL of 1x and 10x cell dilutions was then plated on LB + Ampicillin (50 µg/µL) selection plates.

Sequencing. Potential pTEAM candidate isolates were diluted to 30ng/µL in 15µL solution and sent to Plasmidsaurus for nanopore sequencing. The sequence was read by sensing electrical currents generated as different base pairs pass through nanopores on a flow cell (13). Returned sequencing information was analysed using SnapGene and Geneious Prime.

Whole-Cell Lysate Sample Preparation. The optical density of whole cell samples of UT5600 *Escheria coli* (pENS and pTEAM grown in LB 50µg/mL Ampicillin with or without 0.5mM IPTG) were measured at 600 nm. Protein samples were diluted so that 1mL of each sample had OD600 of 1 (approximately 8 x 10^8 coli cells per mL), then pelleted by centrifugation (10,000 RPM for 3 minutes) and resuspended in 50 uL of PBS. Protein samples were thoroughly mixed and diluted with 2X Laemmli Sample Buffer with 5% BME buffer, for final concentrations of 2X and 5X. Samples were denatured at 95°C for 10 minutes and cooled on ice for 15 minutes.

SDS-PAGE Electrophoresis and Membrane Transfer. Protein samples were diluted with 2X Laemmli Sample Buffer with 5% BME buffer, for final concentrations of 2X and 5X. Samples were denatured at 95°C for 5 minutes and cooled on ice. 8 µL of each sample was loaded into 4–20% precast mini-PROTEAN® TGX Stain-Free SDS-PAGE gels (BioRad), along with 3 µL of PageRuler™ Plus Prestained Protein Ladder (ThermoFisher Scientific). Protein gels were run at 150 V in 1X TRIS-base/Glycine/SDS running buffer at 4 °C, until adequate separation after 90 minutes. SDS-PAGE gels were visualized using ChemiDoc stain-free protein imaging to confirm equal protein loading. Resolved protein gels were loaded into the TransBlot® Turbo™ Transfer System (BioRad) for 7 minutes until complete transfer onto BioRad nitrocellulose membranes (Trans-Blot Turbo Midi Nitrocellulose Transfer Pack).

Western Blot. In accordance with the Bio-Rad Protein Blotting Guide procedures, nitrocellulose membrane was rinsed with Tris-buffered saline (TBS-T, 0.05% Tween 20) on a blot tray for 5 minutes and blocked for 1 hour at 4 °C. 6X His-Tag antibody (Introvitrogen) was used at a dilution of 1:2000 in 3% BSA solution and incubated with the membrane overnight at 4 °C with agitation (14). Membrane was washed in TBS-T for 5 minutes with agitation for 5 repetitions, then incubated in goat-anti-mouse-IgG HRP-conjugated secondary antibody (diluted 1:10000 in 3% BSA solution) for 2 hours at 4 °C with agitation. Membrane was washed 5 times for 3 repetitions, incubated in Clarity™ Western ECL substrate for 5 minutes, and imaged using the ChemiDoc Imaging System (Bio-Rad).

RESULTS

Construction of pTEAM plasmid. The pDO6935-derivative pENS (Epitope N-Terminal Sequence) plasmid, engineered by Goh et al. (15), was chosen as our vector plasmid as it contains the 6X His-tag at the N-terminus of BrkA necessary for subsequent BrkA expression visualization in cell culture. To determine the insertion point on our pENS vector, restriction sites non-disruptive to the *lac*, *ampR*, or *brkA* genes were found and the HindIII restriction site lying between the BrkA and AmpR regions on pENS was decided upon for our insert site. Primers containing 19bp sites flanking the $lacI^Q$ region of pHN678 attached to 20-bp-long regions homologous to the pENS sequence including 5 of the 6 bases of the HindIII restriction site were designed. Using our primers (Table 1), $lacI^Q$ was amplified using PCR from pHN678 isolates and an agarose gel was used to confirm $lacI^Q$ amplification, as seen by the thick bands at 1200bp (actual: 1214bp; Supplementary Figure S1). Next, pENS was linearized with a HindIII-HF digest and immediately followed up with Gibson Assembly to ligate the pENS vector and $lacI^Q$ insert to create pTEAM plasmid (Figure 1).

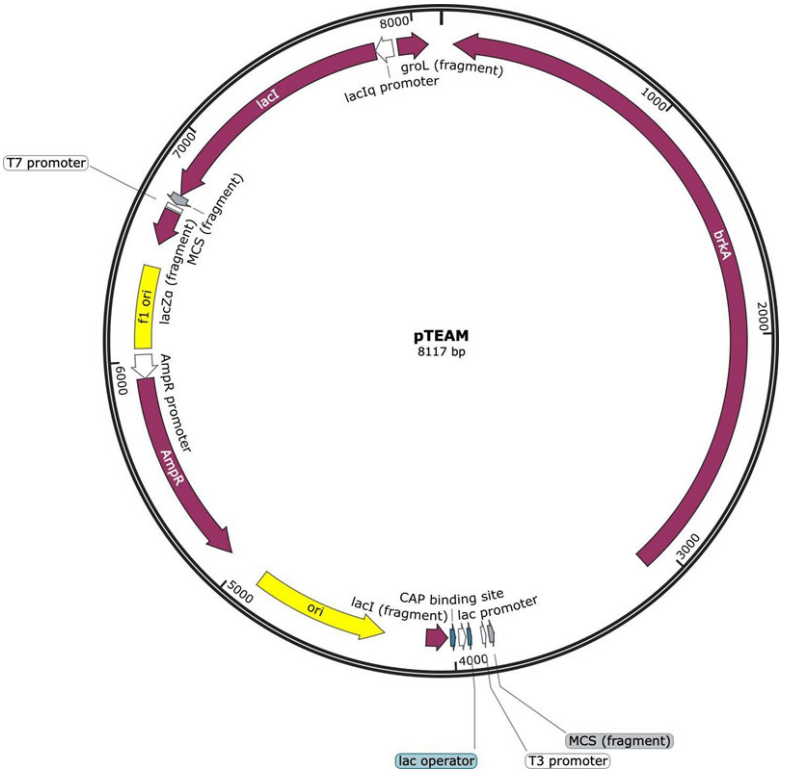

FIG. 1 Confirmed construction of pTEAM plasmid. Vector map of pTEAM plasmid containing pENS-derived *ampR, brkA,* and *lac* operon and pHN678-derived *lacI* experimentally confirmed by nanopore sequencing.

Constructed pTEAM plasmid sequence was confirmed via PCR and sequencing. PCR confirmation of successful Gibson assembly was done using the same primers as above on plasmids isolated from several candidate colonies, then run on agarose gel for visualization. Two potential pTEAM constructs with the cleanest bands at the expected sizes and minimal contamination were sent to Plasmidsaurus for sequencing. The first sample had sequencing results showing a single dominant read at 8,117bp, suggesting that the isolated plasmid contained a single consensus sequence matching the included FASTQ file which was identical to the predicted pTEAM construct constructed in SnapGene (Supplementary Table S1). The other sample was shown to contain this same construct in addition to a second less frequent 2,719bp mystery sequence containing only a promoterless $lacI^{Q}$ fragment and a complete *ampR* gene. Since the first sample showed a clean and correct pTEAM construct, it was selected as the final pTEAM construct going forward.

pTEAM shows no detectable change in BrkA expression when induced with IPTG, confounded by the lack of BrkA expression in the pENS samples. In order to determine the change in BrkA induction in the new pTEAM model, protein isolates were collected from pENS- and pTEAM-transformed UT5600 *E. coli* grown overnight in LB media or LB media containing 0.5mM IPTG, and BrkA expression was quantitatively compared in pENS and pTEAM using Western blot against the 6X His-tag. Total protein quantification using ChemiDoc stain-free imaging revealed even protein loading across conditions (Supplementary Figure S2). Notably, there were darker bands around 40 kDa in protein isolates from pTEAM treated both with and without IPTG (Supplementary Figure S2), supporting the constitutive expression of 42 kDa LacI in pTEAM-transformed *E. coli*. The Western blot detected a thick band just below the 130 kDa marking and another even thicker band under the 100 kDa ladder marking in all of the pTEAM samples, with an extremely faint 130 kDa band found in the pENS sample without IPTG (Figure 2). Despite the inconsistencies in protein ladder travel, these readings roughly align with the expected 103kDa unprocessed BrkA band and 73 kDa processed BrkA band respectively. An unknown protein band just below the 35 kDa ladder marking was visualized in all samples with relatively even abundance.

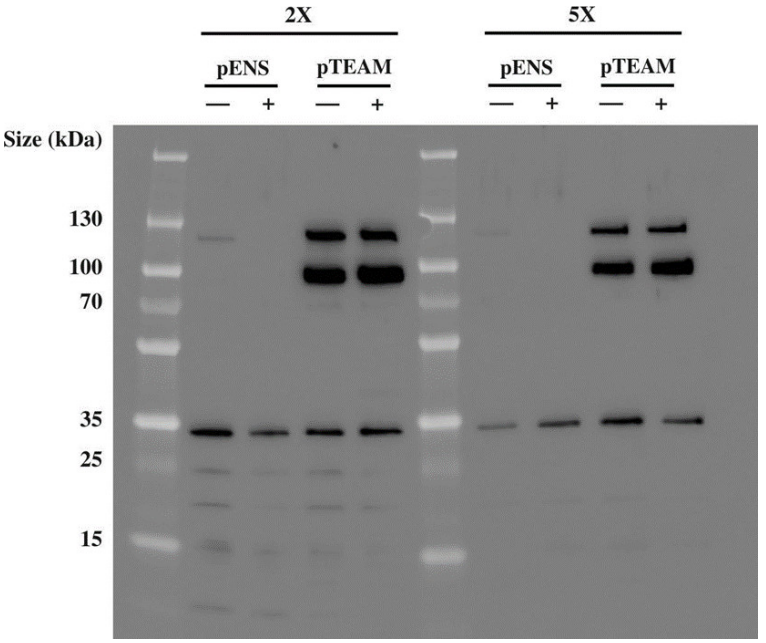

FIG. 2 pENS does not express detectable levels of BrkA expression, while pTEAM demonstrates BrkA expression regardless of IPTG presence. UT5600 *E. coli* cells were transformed with either pENs or pTEAM plasmid constructs, and detection of BrkA with a 6X His tag was conducted with Western blotting. Cells were grown overnight in LB-AMP without or with allolactose isopropyl ß-D-1-thiogalactopyranoside (IPTG), labelled respectively with minus (-) or plus (+) symbols. Bacterial cell lysates were analyzed at concentrations of 2X or 5X.

DISCUSSION

In our study, we aimed to create a pENS-*lacI*^{Q} construct, named pTEAM, to create an IPTG-inducible BrkA expression system in *E. coli* models. Previous studies have implicated the *lac* promoter in regulating BrkA expression within the pENS plasmid, as insertion of the *lac* promoter into promoterless GFP vectors was sufficient for GFP expression in *E. coli* (6). As such, the insertion of *lacI*^{Q} into pENS was predicted to increase LacI expression allowing for better *lac* operon repression and, subsequently, repression of BrkA expression. Contrary to our hypothesis, we observed markedly higher BrkA expression in UT5600 *E. coli* transformed with pTEAM when compared to pENS, with the latter showing negligible expression. IPTG induction also caused no discernable change in BrkA expression in pTEAM-transformed *E. coli*, and contrary to our hypothesis, lower BrkA expression in pENS-transformed *E. coli*.

While it is possible that the 100 kDa and 130 kDa bands are the BrkA passenger domain and uncleaved BrkA respectively, both proteins are 30 kDa above their expected size. As such, it is theorized that an unknown 30 kDa protein may be interacting with BrkA's 73 kDa passenger domain to result in the 100kDa and 130kDa bands. This could potentially be a protein necessary for BrkA post-translational modification and transportation past the inner membrane, or an unexpected protein expressed by UT5600 *E. coli*. Interestingly, there is also an unidentified 30 kDa protein imaged by the anti-6x His antibody under all growth conditions, yet it is unknown whether this is the cause of the 30kDa increase in BrkA size, as well as why it is able to bind the anti-6x His antibody on its lonesome. Further exploration on this protein and its identity should be performed, as it may reveal mechanisms for BrkA processing, transport, and expression in UT5600 *E. coli*.

There are some plausible explanations for the minimal to no BrkA expression in UT5600 *E. coli* transformed with pENS, despite having observed high expression from cells transformed with pTEAM. The most likely explanation is that the pENS plasmid does not allow for the expression of BrkA. This could be due to a variety of factors such as mutations within the BrkA promoter sequence, pENS instability altering accessibility of the BrkA promoter, or increased BrkA toxicity due to the 6X His insertion into BrkA. Notably, previous works have successfully detected BrkA in pDO6935-transformed cells (3, 16). However, it is unclear how the insertion of *lacI*^{Q} into pENS could have seemingly restored BrkA expression. A possible explanation for this could be that the inserted *lacI*^{Q} promoter induced synthesis of full-plasmid mRNA transcripts that allowed for BrkA protein expression, despite the substantial distance between the two genes rendering this unlikely.

The lack of BrkA expression could also be due to issues when expressing recombinant proteins. It is known that the expression of a protein outside of its usual host organism may lead to the formation of protein aggregates or inclusion bodies due to differences between host

protein modifications, trafficking, and expression (17). As such, the lack of BrkA expression in pENS-transformed *E. coli* may be explained by such differences. However, as pTEAM-transformed *E. coli* demonstrated BrkA expression, it is unlikely that the *E. coli* cellular machinery was insufficient for BrkA expression in pENS-transformed cells.

Another plausible explanation could be the presence of a plasmid with no *brkA* gene conferring ampicillin resistance to *E. coli* thought to be transformed with pENS during western blotting. After sequencing two separate pTEAM-transformed colonies post-Gibson Assembly, it was found that one sample contained both our properly assembled 8,117bp pTEAM construct and another 2,719bp mystery construct consisting of a promoterless *lacI*^{Q} fragment and a complete *ampR* gene. We speculate that this plasmid was likely the result of improper HindIII-HF digest and/or Gibson Assembly during pTEAM construction. However, although we visualized pENS plasmid isolates at the expected band size in previous cultures, we could not directly confirm a single pENS presence in the culture used for protein isolation and western blotting. The lack of BrkA expression may therefore be explained by the replacement of pENS with this AmpR-containing fragment plasmid; however, this is unlikely as other studies using the same pENS source plate were able to confirm pENS's purity via sequencing, and the isolated plasmids from our transformed pENS sample matched the expected pENS length.

Separate from from the inconsistencies in pENS BrkA expression, we concerningly found IPTG-independent expression of BrkA in the pTEAM transformed E. coli, as well as higher BrkA expression in pENS without IPTG induction indicating that BrkA synthesis may not be regulated by the *lac* operon in pENS. This finding is also consistent with concurrent findings from our lab by Lin et al. (18), who also found no difference in BrkA expression in pDO6935 under multiple IPTG conditions. Though the study by Haniak et al. indicated that the upstream *lac* promoter region was sufficient for GFP expression in a promoterless vector (6), our findings support Sidhu et al. in their implication of other sequences in the synthesis of BrkA (19). However, we were never able to confirm the expression of LacI from our pTEAM construct, meaning that the consistency in BrkA expression between treatment conditions could be from a lack of repressor expression opposed to IPTG induction, and further studies would be required to support this experimental result.

Conclusions To summarize, we created a new pTEAM plasmid construct based on the pENS plasmid with an added *lacI*^{Q} gene. This addition did not appear to alter the IPTG induction capabilities of the new system for the pTEAM construct showed generally high expression of BrkA compared to the base plasmid under all conditions. This suggests that there may still be information missing about the promotion mechanisms of BrkA in the pDO6935 *E. coli* model. With further validation of these data, our model may provide a potentially useful tool for elucidating the role of LacI and the *lac* operon in pDO6935 BrkA expression.

Future Directions During this study, the pENS construct was used as a visual control for quantifying the baseline expression of BrkA in pDO6935, but potential problems were noted with either the detection of BrkA or expression of it from this construct which was not expected for our experimental results. In order to support our findings, replication of these experiments with an alternative expression vector or tag for visualization should be used in order to ensure that the baseline expression of BrkA in the pDO6935 model is intact in the experimental model including with and without IPTG followed by addition of *lacI*^{Q} and validation of the experimental results regarding the addition of *lacI*^{Q} on BrkA expression. Future studies confirming increased LacI expression in pTEAM-UT5600 cells either qualitatively via immunoblot or quantitatively via Bicinchoninic acid (BCA) assay are necessary to ensure that LacI is being expressed to a sufficient extent above baseline to have an impact on the number of *lac* operons found in cells with pDO6935-based plasmids. If LacI is expressed at higher levels in pTEAM against pENS controls and subsequent immunoblots demonstrate similarly high levels of BrkA expression in pTEAM, there may be a biological or transcriptional explanation for our data, necessitating future studies to look into the interaction between LacI and the BrkA operator. If LacI is found to not be expressed in pTEAM there could be transcriptional errors in our product, and a different insertion point on pENS or alternative method for lacI expression could be explored to ensure sufficient LacI expression levels for *lac* operon repression.

Additionally, the impact of *lacI*^{Q} on BrkA expression via the *lac* promoter could be explored by cloning both *lacI*^{Q} and the pDO6935 *lac* operon region upstream of a promoterless vector, such as pLISA created by Haniak et al., to ensure proper LacI expression and function within vectors.

To account for the lack of BrkA expression seen in pENS, a different 6X-His tagged pDO6935 derivative could be used instead, such as the pPALMC1 construct, as it has been reported to demonstrate proper visualization on immunoblots more consistently than pENS.

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