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Preliminary Steps Toward Establishing a Two-Plasmid System to Drive *Lactobacillus plantarum* Promoter Expression in *Escherichia coli* Using *Lactobacillus plantarum* Sigma Factor, RpoD

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SUMMARY The use of bacterial genes in biotechnology is widespread, but the precise functions of many genes belonging to environmental microbes remain unknown in function, as methods to culture many bacteria are yet to be optimized. To induce the expression of heterologous genes from unculturable bacteria, Gaida *et al.* introduced a two-plasmid system to drive heterologous promoter expression using the *Lactobacillus plantarum* sigma factor, RpoD, in *Escherichia coli*. This successfully resulted in increased expression of the *Lactobacillus plantarum* metagenomic libraries inserted into *E. coli*. Given the promise of this technique, we aimed to set up this two-plasmid system in our laboratory. In this paper, we describe key features of the two-plasmid system and progress with respect to transforming and propagating the plasmids in *E. coli* host strains. The two-plasmid system consists of a *L. plantarum* sigma factor expression vector, pLPLσ, and a second plasmid, pLR-GFP, responsible for carrying heterologous promoters while also providing a method to quantify heterologous promoter expression via a promoter-gfp trap concept. Prior to the insertion of heterologous promoters, pLR-GFP serves as the promoterless-GFP trap destination vector. The pUC-LR-GFP destination vector is near identical in construction to pLR-GFP, but differs only in the presence of a lac promoter located upstream of the segment where heterologous promoters are later inserted, such that the heterologous promoter-gfp trap segment are under its control, and when induced will result in transcription and GFP expression. pUC-LR-GFP thus acts as a positive control and validates the promoter-gfp trap concept. pControl is the negative control for pLPLσ, with near identical construction as pLPLσ, but lacking *rpoD.* We were able to successfully propagate pControl and pLPLσ in *E. coli* DH5α. However, due to the presence of the toxic *ccdB* gene in pLR-GFP, it was instead propagated in the *ccdB* resistant *E. coli* JM109 strain. We also performed restriction digest analysis of each plasmid, and confirmed the identity of pLPLσ via Sanger sequencing. Future steps to fully establish this two-plasmid system in the laboratory include utilizing the Invitrogen Gateway technique to clone heterologous promoters into pLR-GFP, co-transformation of pLPLσ and pLR-GFP into *E. coli* DH5α, and using flow cytometry to quantify and analyze GFP expression in the presence or absence of RpoD.

INTRODUCTION

Investigating the function of genes in bacterial genomes can lead to the development of new tools in biotechnology (1). With the function of one-third of all protein coding genes from bacterial genomes still unknown, there is potential to advance the field of biotechnology by identifying novel enzymes, proteins, and biological pathways present in metagenomic libraries and harnessing their power through genome engineering (2). However, the methods to culture many bacteria have yet to be optimized, making it difficult to investigate their protein function in a laboratory setting. One way to study the function of a gene is via heterologous expression of genes from foreign environmental microbes in culturable host organisms, and their subsequent biochemical and phenotypic characterization (3, 4). Genetic material extracted from the environment can be inserted into the genomes of culturable

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bacteria, such as *Escherichia coli*, enabling access and the functional screening of metagenomic libraries (1, 5). A significant barrier to this technique, however, is that host screening organisms often lack the appropriate cellular machinery required to transcribe heterologous genes, resulting in low heterologous promoter expression that restricts their use in biotechnological settings (5). In bacteria, the RpoD housekeeping sigma factor plays a major role in carrying out promoter recognition by recruiting RNA polymerase to initiate transcription, and is therefore required for the transcription of many essential genes. Recent studies have begun experimenting with the expression of heterologous sigma factors in *E. coli* to improve the transcriptional efficiency of heterologous promoters (6). For example, this can be seen in the use of cyanobacterial *Anabaena* sigma factors to increase heterologous expression of cyanobacterial gene clusters in *E. coli* (7). Other studies have employed the use of shuttle vectors to heterologously express metagenomic libraries, and broaden the host range for functional screening (8).

Gaida et al. designed a two-plasmid system capable of increasing heterologous promoter recognition, upon expression of heterologous sigma factors in *E. coli* (Fig. 1). This enables a more efficient strategy to functionally screen metagenomic libraries (5). In their experiment, *E. coli* was transformed with two plasmids: pLPLσ, which expresses the RpoD sigma factor of *Lactobacillus plantarum* (5), and a second plasmid containing an *L. plantarum promoter insert* fused upstream of a *GFP* gene (Fig. 1A). Expression of RpoD will result in increased transcription of the *L. plantarum* promoter and *gfp* gene. The resulting fluorescence intensity serves as a method to directly quantify how heterologous promoter expression changes in the presence or absence of RpoD (Fig. 1B). *E. coli* successfully integrated the sigma factor into its own cellular machinery and expressed heterologous *L. plantarum* genes, which could not otherwise be expressed.

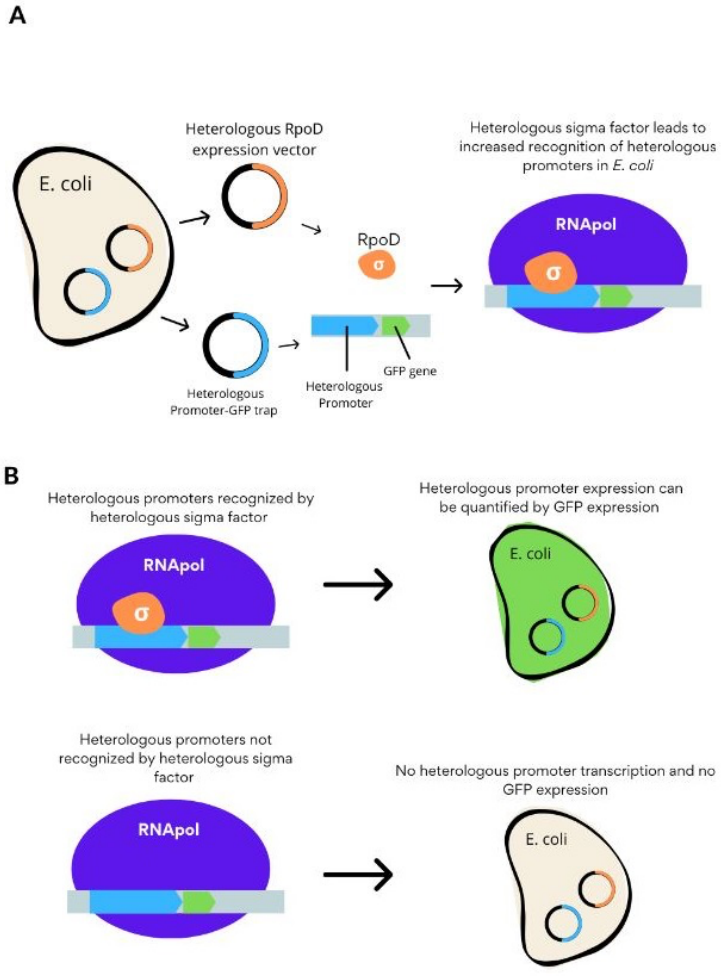


FIG. 1 Experimental design and concept. Adapted from Gaida et al. (5) **A)** *E. coli* is transformed with a two-plasmid system to enable heterologous promoter expression using a heterologous sigma factor, RpoD. The first plasmid is responsible for expressing a heterologous sigma factor, RpoD. The second plasmid is responsible for carrying heterologous promoters, and providing a method to quantify heterologous promoter expression via GFP expression. Upon expression of RpoD, it recruits the host cell RNA polymerase, leading to increased recognition of heterologous promoters. **B)** The heterologous promoter is placed directly upstream of a GFP gene. Heterologous promoter recognition due to the presence of a heterologous sigma factor, leads to its transcription and GFP expression. Should the heterologous sigma factor fail to recognize the heterologous promoter, transcription and GFP expression will not occur (5).

To replicate the experiment conducted by Gaida et al. we obtained their original plasmids (Supplementary Fig. 1) and plasmid maps, and successfully transformed and propagated pLPLσ and pControl in *E. coli* DH5α. pLR-GFP, however, failed to propagate in *E. coli* DH5α due to the presence of *ccdB.* Upon retrieval of the ccdB resistant *E. coli* JM109 strain, we successfully transformed and propagated pLR-GFP, while also showing a proof of concept of the function of *ccdB*.

We then tested the transformed *E. coli* under various growing conditions to investigate which would optimize plasmid yield. Restriction enzyme digestion, followed by agarose gel electrophoresis, was utilized to gain insight into the identity of the obtained plasmids. Finally, the plasmids were sent for Sanger sequencing. In this project, several key insights were gained regarding the plasmids retrieved, including confirmation of assembly and design of pControl, pLR-GFP and pLPLσ, the role of *ccdB* and proof of its concept, a method to propagate *ccdB*-containing pLR-GFP using JM109, and confirmation of the identity of pLPLσ via Sanger sequencing.

METHODS AND MATERIALS

Preparation of Ampicillin and Chloramphenicol stock solutions. To prepare a stock solution of ampicillin, 100 mg of sodium ampicillin was dissolved per 1 mL of H_2O, and subsequently sterilized by passing the solution through a sterile 0.22µm filter. To prepare a stock solution of chloramphenicol, 25 mg of chloramphenicol was dissolved per 1 mL of 100% EtOH, and filter-sterilized as outlined for ampicillin. The stock solutions were further diluted to a working concentration of 100 ug/mL in dH2O for ampicillin, and 25 ug/mL in 100% EtOH for chloramphenicol.

Preparation of LB broth with ampicillin and/or chloramphenicol. To achieve the working concentrations for ampicillin and chloramphenicol outlined above, stock solutions were added to LB broth at a ratio of 1 µL of stock solution for every 1 mL of broth desired. To prepare 100 mL of LB broth with ampicillin or chloramphenicol, 100 µL of stock ampicillin or chloramphenicol solution was added to 100 mL of LB broth. For LB broth with ampicillin and chloramphenicol, 100 µL of each antibiotic was added to 100 mL of LB broth. The LB broth with ampicillin and/or chloramphenicol was prepared fresh for every experiment.

Preparation of calcium chloride solutions for chemical competency. In order to make chemically competent *E. coli* cells, we prepared calcium chloride solutions using the protocol from Chang et al. outlining the preparation of calcium competent cells, and heat-shock transformation as a reference (9). Following their guidelines, 1M $CaCl_2$, 0.1M $CaCl_2$, and 0.1M $CaCl_2$ + 15% glycerol solutions were prepared. For the 1M $CaCl_2$ stock solution, 11.1g of anhydrous $CaCl_2$ was mixed with 80 mL of dH_2O until fully dissolved and topped up to 100 mL with more dH_2O. For the 0.1M $CaCl_2$ solution, 10 mL of 1M $CaCl_2$ was added to 90 mL of dH_2O. For the 0.1M $CaCl_2$ + 15% glycerol solution, 6 mL of 1M $CaCl_2$ was mixed with 9 mL of glycerol, and 45 mL of dH_2O. All solutions were sterilized by autoclaving.

Preparation of chemically competent *E. coli* DH5α. In order to make *E. coli* DH5α competent cells, we used the Chang et al. protocol mentioned above as a reference (9). To obtain an overnight culture, we inoculated 1 mL of LB with a pure colony of *E. coli* DH5α, and shake incubated it at 37°C, 200 rpm for 12-16 hours. After incubation, 1 mL of the overnight culture was added to 99 mL of LB broth and left in the shaking incubator. We periodically retrieved OD readings using a UV-Vis spectrophotometer until the cells reached an OD of approximately 0.4. The 100 mL culture was then split into four ice-cold Oakridge tubes, which were then incubated on ice for 20 minutes, and then centrifuged at 4000rpm for 10 minutes at 4ºC. The supernatant was discarded by tipping the tubes over a discard bin, and then pipetting off any remaining media. Each pellet was then resuspended with 20 mL ice-cold 0.1M $CaCl_2$, and incubated on ice for 30 minutes. The tubes were then centrifuged at 4000 rpm for 10 minutes at 4ºC. The supernatant was discarded, and the pellets were combined and resuspended in 5 mL of ice-cold 0.1M CaCl2 + 15% glycerol solution. The

competent *E. coli* DH5α cells were separated into 50 µL aliquots and stored in the -80ºC freezer, unless they were used for downstream transformation the same day.

Initial plasmid propagation of pLPLσ, pControl, pLR-GFP, and pUC-LR-GFP in competent *E. coli* DH5α. In order to build a two-plasmid expression system to test the expression of heterologous promoters in *E. coli* DH5α cells, we requested pLPLσ, pControl, pLR-GFP, and pUC-LR-GFP from Dr. Eleftherios Papoutsakis from the Department of Chemical and Biomolecular Engineering at the University of Delaware. The plasmids arrived in filter papers, and were eluted in 20 µL of Elution Buffer, which was obtained from the Invitrogen PureLink Quick Plasmid Miniprep Kit from Fisher Scientific. For all of the transformations performed in this project, we followed the Chang et al protocol as a reference (9). For each of the plasmids, a mixture of 1-5 µL of plasmid DNA and 50 µL of competent *E. coli* DH5α solution was prepared, and incubated on ice for 30 minutes. The mixture was placed in a 42ºC water bath for 30 seconds, and then placed on ice for 2 minutes. After heat-shock, 1 mL of pre-warmed LB was added to each vial, and they were further incubated in a shake incubator at 37ºC and 200 rpm for 1 hour. The transformed cells were diluted in 1:10, 1:100 and 1:1000 concentrations, and plated, along with undiluted cells, on a series of LB agar plates containing ampicillin. For the negative control, we spread untransformed competent *E. coli* DH5α into ampicillin LB agar plates of the same antibiotic concentration.

Propagation of pLR-GFP in *E. coli* JM109, a ccdB resistant strain. Two ccdB resistant *E. coli* strains, XL-1 Blue and JM109, were obtained from Dr. Hayedeh Behzad, a research associate at the Weng Lab, from the Terry Fox Laboratory department at BC Cancer Research Centre. To obtain two overnight cultures, we inoculated 1 mL of LB with a single colony of XL-1 Blue, and another 1 mL of LB with a single colony of JM109. The cultures were placed in a shaking incubator at 37°C and 200 rpm for 12-16 hours. Both cell types were subcultured by adding 1 mL of overnight culture to 99 mL of LB broth and then shake incubated overnight. From this step onwards, only the JM109 culture was kept. The remaining steps of this procedure were the same as those described in the chemical competency and transformation protocol outlined above for DH5α cells. The JM109 cells transformed with pLR-GFP were diluted in 1:10, 1:100 and 1:1000 concentrations and plated, along with undiluted cells, on a series of LB agar plates containing working concentrations of either ampicillin only, ampicillin + chloramphenicol, or chloramphenicol only. For the negative control of each antibiotic, untransformed competent JM109 cells were spread onto LB agar plates containing the respective antibiotic concentration.

Plasmid extraction of pLPLσ, pControl and pLR-GFP. Isolated colonies from ampicillin LB agar plates containing *E. coli* DH5α transformed with pLPLσ, *E. coli* DH5α transformed with pControl, and JM109 transformed with pLR-GFP, were selected, and each was used to separately inoculate 2 mL of LB broth with the appropriate antibiotic (Table 1). The inoculates were incubated at 37ºC overnight, and plasmid purification was performed using the Invitrogen PureLink Quick Plasmid Miniprep Kit from Fisher Scientific. For all extractions, an aliquot of TE Buffer was prewarmed to maximize plasmid DNA yield (10). The plasmid concentrations obtained were analyzed using the NanoDrop 2000 Spectrophotometer from Thermo Fisher Scientific. All plasmids were stored at -20°C in 10 µL aliquots for downstream usage.

Verification of plasmid construct via restriction digest analysis. To confirm the size and identity of the isolated plasmids pLR-GFP, pLPLσ and pControl, we performed two restriction digest analyses using agarose gel electrophoresis (11, 12). For both analyses, the steps outlined here were implemented. For details regarding the differences between the two gels, see the discussion section. To make the gel, a 1% agarose solution was prepared in TBE buffer. The solution was then microwaved for 1-3 minutes, until the agarose was completely dissolved and allowed to cool to approximately 50ºC. 5 µL of RedSafe was added to the cooled solution, and it was then poured into a gel tray with the well comb in place. The gel was allowed to sit overnight at 4ºC. All of the enzymes used for restriction digest analysis were provided by New England BioLabs. For the restriction digest of pLR-GFP, XbaI and

TABLE. 1 Antibiotic resistance conferred by pLPLσ, pControl, pUC-LR-GFP and pLR-GFP.

Plasmid	Antibiotic resistance
pLPLσ	Chloramphenicol
pControl	Chloramphenicol
pUC-LR-GFP	Ampicillin + Chloramphenicol
pLR-GFP	Ampicillin + Chloramphenicol

XmnI Time-Saver restriction enzymes were used. For the restriction digest of pLPLσ, the restriction enzymes AfeI and HindIII were chosen. For the restriction digest of pControl, Time-Saver XmnI and BspHI were the restriction enzymes implemented. All incubations were performed at 37ºC. Besides preparing the reaction mixes outlined above, a control sample of enzymes only as well as a control sample of undigested plasmid were individually prepared for pLR-GFP, pLPLσ and pControl. To visualize the results, the samples were then prepared for gel electrophoresis. Each sample was stained with MassRuler DNA Loading Dye (6X) to a final concentration of 1X per sample. The electrophoresis unit was assembled and filled with 1X TBE buffer until the entire gel was covered. The ready-to-use O'GeneRuler DNA Ladder Mix was loaded for molecular weight comparison purposes. Once all the samples were loaded, the gel was run for 1.5 hr at 120V. Bands were then visualized under UV light by using ChemiDoc MP Imaging System from BioRad.

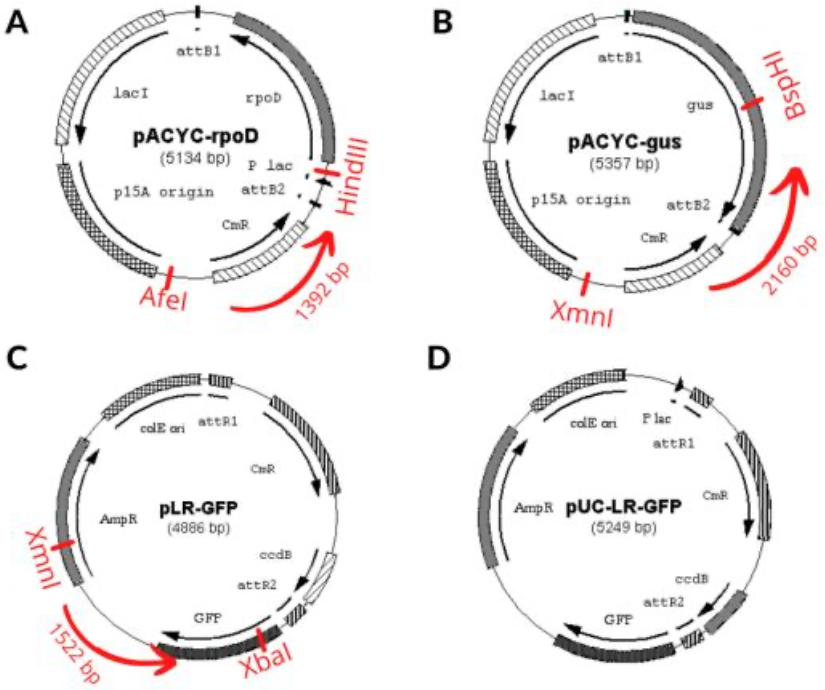
Optimization of plasmid yield of pLPLσ and pLR-GFP. We attempted to optimize plasmid yield of pLPLσ and pLR-GFP by altering yeast concentrations in the LB medium, and the temperature of the overnight incubation before plasmid extraction (13). Two types of LB media were prepared, the usual recipe, which included 5 g of yeast extract, and LB24 which used 24 g of yeast extract (14, 15). The two temperatures tested for overnight shaking incubation were 37°C and 42°C. Note that N=1 for each of the established conditions. To set-up the cultures, 5 mL inoculates with either *E. coli* JM109 cells harboring pLR-GFP, or *E. coli* DH5α cells harboring pLPLσ, were left in the shaking incubator at 200 rpm for 12-16 hours overnight. The morning post-incubation, optical density measurements were taken using a UV-Vis spectrophotometer. Plasmid purification was then performed using the Invitrogen PureLink Quick Plasmid Miniprep Kit from Fisher Scientific. The DNA concentration was analyzed using the NanoDrop 2000 Spectrophotometer from Thermo Fisher Scientific. All plasmids were stored at -20°C in 10µL aliquots for downstream usage.

Sanger sequencing. To further confirm the identity of our isolated plasmids as pLR-GFP, pLPLσ, and pControl, we sent our plasmids for Sanger sequencing to GeneWiz, from Azenta Life Sciences, in South Plainfield, New Jersey. No primers were designed for pLR-GFP and pLPLσ, since they were found to contain regions in their plasmid maps compatible with universal primers. pLR-GFP was sequenced with M13Forward and pLPLσ with M13Reverse. A primer for pControl was designed with a GC clamp and a melting point between 55-60°C using Primer3 software (17). The following primer sequence was deemed viable for pControl: 5'-CACCGACATGTGGAGTGAAG-3'. The designed primer sequence was ordered from Integrated DNA Technologies, and sent along with pControl according to the parameters outlined by GeneWiz (18, 19). The sequencing results obtained from GeneWiz were aligned and examined using SnapGene software.

RESULTS

The function of pControl as a negative control of pLPLσ was found through the characterization of pLPLσ and pControl using the plasmid maps provided by Gaida et al. In order to build a two-plasmid expression system to test the expression of heterologous promoters in *E. coli* DH5α cells, we analyzed two of the plasmids designed by Gaida et. al. pLPLσ and pControl. The plasmid, pLPLσ, was constructed from the low copy plasmid pACYC-Duet (Novagen). To do so, a segment of the pACYC-Duet backbone was amplified

including the *p15A* origin of replication, the chloramphenicol resistance gene and the *lacI* repressor. An LR recombination cassette amplified from pDEST40 (Invitrogen) was then introduced into the pACYC-Duet partial backbone, resulting in the pACYC-LR destination vector. *Lactobacillus plantarum*, *rpoD*, was then amplified and introduced into the multiple cloning site of pUC19 in front of a lac promoter. The *rpoD* and lac promoter segment was then amplified and introduced directly into pACYC-LR using the Invitrogen Gateway Cloning Technique. This resulted in the construction of pLPLσ or pACYC-rpoD. In pLPLσ, the sigma factor, RpoD, is under the control of the promoter of the *lac* operon such that *rpoD* overexpression can be activated using isopropylthiogalactoside (IPTG), a known inducer of the *lac* operon. When there is no IPTG present, the *lacI* repressor inhibits *rpoD* expression (20). *p15A* is the origin of the replication of this plasmid, and *cmR*, is the chloramphenicol resistance gene that acts as a selection marker to allow for isolation of bacteria containing the plasmid after transformation (Fig. 2A).



pControl or pACYC-gus was also constructed from the destination vector, pACYC-LR, and is thus a low copy plasmid. However, instead of introducing *rpoD* into pACYC-LR, a promoterless *gus* gene from *Arabidopsis thaliana* was introduced. This was achieved by recombining pENTR-gus vector with pACYC-LR via the Invitrogen Gateway Cloning Technique resulting in pControl.

pControl is almost identical to pLPLσ, and only differs by the presence of *gus* in place of *rpoD* (Fig. 2B). With the absence of *rpoD*, pControl functions to ensure that no other aspect of pLPLσ can be attributed to the elevated expression of heterologous genes seen when IPTG is present. In this way, through the characterization of pLPLσ and pControl using their plasmid maps, it was found that pControl acts as the negative control for pLPLσ, when the co-transformation with the heterologous promoter *gfp* trap takes place (Fig. 3B).

FIG. 2 Maps of plasmids obtained from Gaida et al. (**A**) plasmid map of pLPLσ (pACYC-rpoD). This plasmid contains *L. plantarum rpoD* sigma factor. The lac promoter is located directly in front of *rpoD* such that its overexpression can be easily induced using IPTG. When there is no IPTG present, the *lacI* repressor inhibits *rpoD* expression. The presence of *attB* sites allow for efficient recombination of *rpoD* from any organism using the Invitrogen Gateway technique. CmR encodes chloramphenicol resistance, allowing for selection of the plasmid on chloramphenicol enriched plates. The red labels indicate the relative locations of restriction sites AfeI and HindIII, and their predicted fragment size of 1392 bp. *p15A* is the origin of replication which was amplified, along with *cmR* and *lacI*, from a portion of the pACYC-DUET backbone. (**B**) plasmid map of pControl (pACYC-gus). pControl is near identical in construction as pLPLσ but instead contains a promoterless *gus* gene from *Arabidopsis thaliana* in the place of *rpoD*. pControl thus acts as a negative control of pLPLσ. The red labels indicate the relative locations of restriction sites XmnI and BspHI, and their predicted fragment size of 2160bp. (**C**) plasmid map of pLR-GFP. This plasmid contains *ampR* and *cmR* genes encoding for ampicillin and chloramphenicol resistance, respectively. This allows for efficient selection of the pLR-GFP on ampicillin and chloramphenicol enriched plates. pLR-GFP also contains the *ccdB* gene, which encodes for a bacterial gyrase inhibitor that can causes cell death in susceptible host strains. The presence of *attR* sites allow for efficient cloning of any desired heterologous promoter into pLR-GFP using the Invitrogen Gateway technique. The *gfp* gene is located in front of where the heterologous promoters are meant to be cloned into, allowing for a promoter-*gfp* trap to be assembled. *colEori* is the origin of replication. (**D**) plasmid map of pUC-LR-GFP. pUC-LR-GFP is near identical in construction as pLR-GFP but contains an additional lac promoter. Upon recombination of heterologous promoters into pUC-LR-GFP, promoter will be under the control of the lac promoter which can be induced using IPTG. Expression of the promoter will lead to GFP expression. Thus, pUC-LR-GFP acts as a positive control for heterologous promoter and GFP expression, and validates the proposed promoter-*gfp* trap concept.

The role of pUC-LR-GFP as a positive control of pLR-GFP, and as a validation of the experimental concept was found through the characterization of pLR-GFP and pUC-LR-GFP using the plasmid maps provided by Gaida et al. To further our understanding, we analyzed Gaida et al.'s design of pLR-GFP, and pUC-LR-GFP, and the genes outlined in their plasmid maps (Fig. 2C, 2D). To obtain a promoterless-*gfp* trap destination vector, the *gfp* gene was amplified from pLenti7.3/V5-GW/lacZ (Invitrogen), and cloned into the high copy pUC19 plasmid to be under the control of the promoter of the *lac* operon. An LR-cassette amplified from pDEST14 (Invitrogen) was cloned upstream of *gfp*. This destination vector was designated pUC-LR-GFP. After removal of the lac promoter from pUC-LR-GFP, the destination vector, pLR-GFP, was constructed. In pLR-GFP, *ampR* and *cmR* provide ampicillin and chloramphenicol resistance, respectively, and *colE* is the origin of replication (Fig 2C). In this plasmid, the purpose of the promoterless *gfp* gene is to use the Gateway cloning technique to insert a promoter of our choice upstream of *gfp*. During this technique, the genes located in between the *attR* sites, *cmR*, and *ccdB*, which produces a toxin that results in cell death, are both excised, allowing for bacteria that have successfully integrated the heterologous promoter into the plasmid to be the only ones that survive (21). pUC-LR-GFP is almost identical to pLR-GFP, and only differs by the presence of the promoter of the *lac* operon upstream the *attR* sites (Fig. 2D). In this way, GFP expression does not depend on the promoter inserted during Gateway cloning, and instead, under IPTG induction, GFP will be constitutively expressed. Besides being an ideal positive control, pUC-LR-GFP serves as a validation of the experimental concept, as transcription and GFP expression will still occur despite the absence of heterologous RpoD. Therefore, through the characterization of pLR-GFP and pUC-LR-GFP, it was found that pUC-LR-GFP acts as a positive control of pLR-GFP, and as a validation of the experimental concept.

The Gateway Recombination Cloning Technology provides an efficient method for constructing the GFP trap containing a *Lactobacillus plantarum* promoter. Because pLR-GFP and pUC-LR-GFP were designed to be destination vectors in the Gateway Recombination Cloning Technology from Invitrogen, analysis of this cloning technology is necessary to further our understanding of these plasmids. Gateway cloning is based on two reactions, the BP reaction and the LR reaction. The BP reaction leads to the generation of an entry clone containing the gene of interest flanked by *attL* sites, which is necessary for the LR reaction to proceed (Fig. 3A). The BP reaction takes place between the *attB* sites flanking the gene of interest, and the *attP* sites of a *ccdB*-containing donor vector. This reaction is catalyzed by BP clonase enzyme mix, and there are two products produced, an entry clone containing the gene of interest, and a *ccdB*-containing byproduct (Fig. 3A). The LR reaction takes place between the *attL* sites flanking the entry clone and the *attR* sites of the destination vector. This reaction is catalyzed by LR Clonase enzyme mix, and it leads to the desired expression clone, and the simultaneous excision of the toxin-producing *ccdB* gene from the destination vector. In the two-plasmid system we are interested in constructing, pLR-GFP is the destination vector used to construct the heterologous promoter-*gfp* trap. To do so, an *L. plantarum* heterologous promoter, flanked by *attB* sites, will need to react with a donor vector containing *attP* sites. As shown in Figure 3A, this reaction will be catalyzed by BP clonase enzyme mix, and it will generate the entry clone that contains the *L. plantarum* promoter flanked by *attL* sites, and a *ccdB*-containing byproduct. The entry clone produced will then react with the *attR* sites of pLR-GFP to obtain the desired expression clone containing the heterologous promoter-*gfp* trap segment (Fig. 3A). In this way, the Gateway Recombination Cloning Technology provides an efficient method to construct the GFP trap containing the *L. plantarum* promoter.

pLPLσ and pControl were successfully propagated in competent *E. coli* DH5α. In order to amplify pLPLσ, pControl, pLR-GFP, and pUC-LR-GFP, we transformed them via heat shock into *E. coli* DH5α and subsequently incubated them on plates with the corresponding antibiotic for each plasmid (Table 1). Plates that were spread with *E. coli* DH5a transformed with either pLPLσ or pControl showed growth. However, plates that were spread with *E. coli* DH5α transformed with either pLR-GFP or pUC-LR-GFP showed no growth. Negative controls also showed no growth. These results indicate that pControl and pLPLσ successfully

propagated in *E. coli* DH5α, while the propagation of pLR-GFP and pUC-LR-GFP in *E. coli* DH5α was unsuccessful.

pLR-GFP is hypothesized to be unable to be transformed into *Escherichia coli* DH5α due to the presence of *ccdB*. To further investigate reasons for the unsuccessful propagation of pLR-GFP in *E. coli* DH5α, we reviewed the initial characterization of this plasmid performed based on its plasmid map. In doing so, we discovered that *E. coli* DH5α are ccdB sensitive cells. *ccdB* encodes for a toxin that interferes with the activity of DNA gyrase, which prevents the relaxation of DNA supercoiling during replication. This results in the breakage of plasmid and chromosomal DNA, and thus cell death (22). In pLR-GFP, *ccdB* acts as a negative selection marker to increase cloning efficiency when performing Gateway cloning (21). Being that *E. coli* DH5α cells are susceptible to the ccdB toxin, we hypothesized that the *ccdB* gene prevented our pLR-GFP containing DH5α cells from surviving the transformation.

pLR-GFP was successfully propagated in *E. coli* JM109, a ccdB resistant strain. We asked how we could counteract the effects of *ccdB,* such that cells transformed with pLR-GFP could survive and allow for the propagation of the plasmid for downstream usage. It was found that the *ccdA* gene was required to inhibit the actions of the ccdB toxin (22). To investigate this, we sourced two *ccdB* resistant strains from the Weng Lab at BC Cancer Research Centre, JM109 and XL-1 Blue. These strains contain the F plasmid, which carries *ccdA, a* gene that inhibits the toxin-producing *ccdB* gene (22, 23). When performing chemical competency of XL-1 Blue and JM109, we noticed that growth was fastest in the *E. coli* JM109 strain, and we therefore continued with only this strain for further transformations. JM109 was then transformed with pLR-GFP and streaked onto chloramphenicol, ampicillin, and chloramphenicol and ampicillin enriched plates. Growth was visible on all three plates, with no growth seen on any of the negative controls. These results indicate that the transformation of pLR-GFP into *E. coli* JM109 provided a successful method to counteract the effects of *ccdB,* thus allowing for cell survival and further propagation of pLR-GFP.

The identity of pLPLσ pLR-GFP and pControl are suggested by restriction digest analysis. In order to gain insight into the identities of pLPLσ, pLR-GFP and pControl, we performed a restriction enzyme digestion followed by agarose gel electrophoresis. Due to low plasmid yields, this experiment was performed twice. Each of the plasmids were subjected to three conditions: undigested plasmid, digested plasmid, and the restriction enzymes alone as a negative control. All negative controls showed no bands as expected. All experimental bands derived from digested pLPLσ, pLR-GFP and pControl matched the predicted band sizes obtained from their plasmid maps. These results confirm the relative locations of restriction sites indicated on the plasmid maps and thus highly suggest the identity of pLPLσ, pLR-GFP and pControl.

Altering yeast and temperature conditions may increase plasmid DNA yield of pLPLσ and pLR-GFP. To optimize the plasmid yield of pLPLσ and pLR-GFP, yeast concentration of the LB medium and temperature of the overnight incubation were both altered. The plasmid

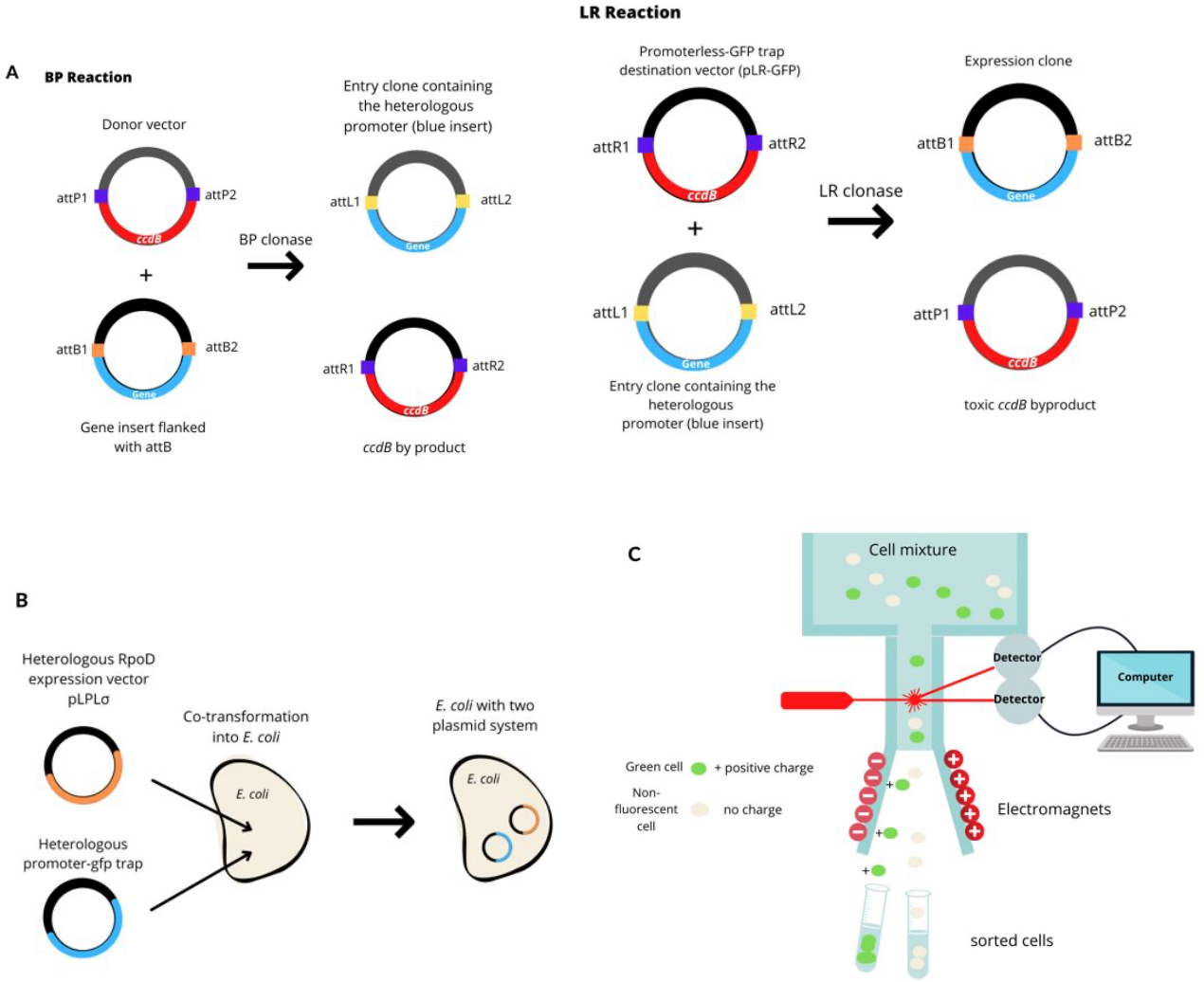


FIG. 3 Steps to establish a two-plasmid expression system to drive heterologous promoter expression using heterologous sigma factors. A) The Invitrogen Gateway technique can be utilized to construct the heterologous-*gfp* trap expression clone. To do so, a BP reaction will need to take place between the *attP* sites of a donor vector and the *attB* sites of a PCR product or expression clone containing a heterologous promoter. This will generate an *attL* entry clone containing the heterologous promoter. This entry clone will then undergo an LR reaction between its *attL* sites and the *attR* sites of pLR-GFP or pUC-LR-GFP, resulting in the construction of an expression vector containing the heterologous promoter-*gfp* trap necessary to the two-plasmid expression system. **B)** Co-transformation of pLPLσ and the heterologous promoter-*gfp* trap expression vector in *E. coli*. *E. coli* cultures transformed with pLPLσ and the heterologous promoter-*gfp* trap expression vector will need to be induced with IPTG for several hours to optimize expression of *L. plantarum* RpoD. **C)** Due to the presence of RpoD, transcription of the heterologous promoter will occur, causing the cell to express GFP. Fluorescence intensity can be quantified in a high throughput fashion via flow cytometry.

DNA concentrations obtained from each condition were analyzed using the NanoDrop 2000 Spectrophotometer (Fig. 4). High yeast conditions for pLPLσ yielded ~3X lower DNA concentrations compared to low yeast conditions for pLPLσ. Incubation at 37°C also yielded slightly lower DNA concentrations for pLPLσ (Fig. 4). This suggests that low yeast conditions coupled with incubation at 42°C may increase plasmid yield for pLPLσ. High yeast concentrations for pLR-GFP yielded slightly lower DNA concentrations compared to low yeast conditions. Incubation temperature of 42°C or 37°C seemed not to have a great effect on DNA concentration of pLR-GFP (Fig. 4). This suggests that using low yeast conditions may be better for increasing the plasmid yield of pLR-GFP. However, with the biological replicate for each condition only being N=1, and the lack of statistical power, results from

this experiment are not to be extrapolated in future experiments. Because low yeast conditions may increase plasmid yield for pLR-GFP, and this condition coupled with incubation at 42°C may increase plasmid yield for pLPLσ, it is therefore suggested that altering yeast and temperature conditions may increase plasmid DNA yield of pLPLσ and pLR-GFP.

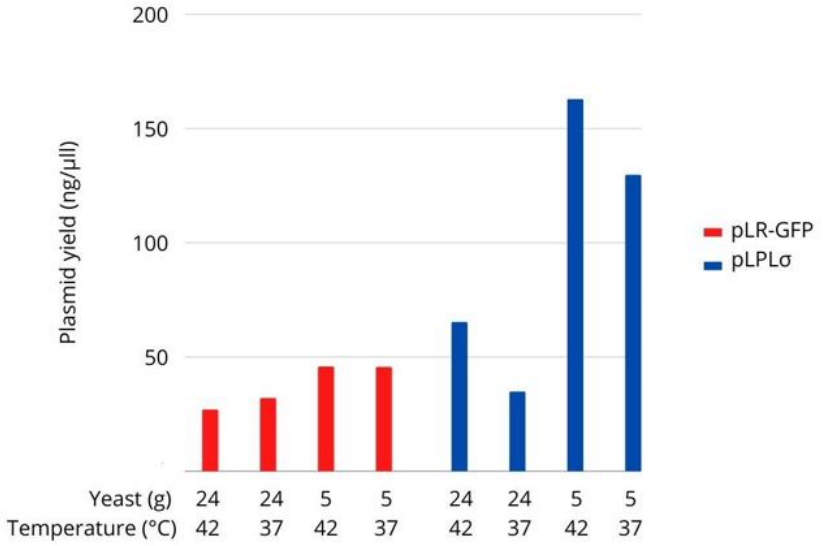


FIG. 4 Altering yeast and temperature conditions may increase plasmid yield of pLPLσ and pLR-GFP.

Identity of pLPLσ was confirmed by Sanger sequencing. To gain further insight regarding the identity of pLR-GFP, pLPLσ, and pControl, Sanger sequencing was conducted. The pControl and pLR-GFP Sanger sequencing results were inconclusive. In contrast, sequencing results for pLPLσ showed nearly complete alignment with a sequence in close proximity to the M13 Reverse primer, with the exception of one mismatch and one gap. These results strongly confirm the identity of pLPLσ.

DISCUSSION

Through the experiments outlined in this paper, we aimed to begin the process of replicating the two-plasmid system used by Gaida et al. in an undergraduate laboratory. This was done by obtaining the plasmids and plasmid maps from their original creators, propagating them, and verifying their identity by enzyme digestion and Sanger sequencing. This led to us learning the effects of the gene *ccdB*, finding a way to propagate the plasmids despite the presence of *ccdB*, and confirming the identity of pLPLσ.

The effects of *ccdB* and a means of propagating pLR-GFP. Upon investigating the plasmid maps, we found that pLR-GFP contains the gene *ccdB*, encoding a bacterial gyrase inhibitor. This leads to replication inhibition in host bacteria, unless the host has the gene *ccdA*, the "antidote" gene for *ccdB*. This gene is used in the Gateway cloning technique used by Gaida et al. but will inhibit the propagation step of a non-resistant host. We obtained a strain of *E. coli* called JM109 which contains *ccdA*. Once made competent and transformed, the JM109 cells acquired resistance to both ampicillin and chloramphenicol. It appears that the JM109 cells have successfully integrated pLR-GFP, which granted the bacteria the resistance to the antibiotics. This also suggested that *ccdB* was responsible for our previous failed transformations of pLR-GFP, as JM109, a ccdB-resistant strain could support the plasmid where DH5α could not. pLR-GFP likely also contained similar genes as those seen in the plasmid map, including the *ccdB* gene, and the antibiotic resistance genes for chloramphenicol, ampicillin. This information serves as evidence towards its identity being pLR-GFP, or at least very like it.

Evidence for the identity of pControl, pLPLσ, and pLR-GFP from restriction enzyme digestion. To gain insight into the structure of the plasmids, and to see if they

correspond to those seen in the accompanied plasmid maps, we performed an enzyme digest reaction, and ran the digested products through an agarose gel (Fig. 3A). The lanes containing digested plasmids pControl and pLR-GFP yielded bands where we expected them, indicating that the plasmids are similar in structure to those in their corresponding plasmid maps. However, the lanes containing digested pLPLσ show only one of the expected bands, at ~ 3742 bp, and they are very faint. Additionally, the undigested plasmid lanes were empty for all conditions, as seen in lane B, for pControl, lane E, for pLPLσ, and lane I, for pLR-GFP (Fig. 3A). The faint DNA bands seen in lanes F and G, for pLPLσ, are likely due to the low plasmid concentration (~ 6-8 ng/ul) that was loaded in each lane (Fig. 3A).

The missing bands could potentially be due to three reasons. The low DNA concentration loaded could have made the bands too faint to see. The bands could be hidden by the haze caused by the gel thickness combined with the positive nature of RedSafe causing its accumulation at the top of the gel. Finally, the break in the gel between 1.2 kb and 1.5 kb could have hidden the band. Due to all of the issues encountered in the first gel run, the plasmids were then subjected to another restriction digest analysis and run in a second gel.

In the second gel (Fig. 3B) lane B shows the digested pLR-GFP, and lane C shows the undigested pLR-GFP. Similarly, the digested pLPLσ can be seen in lane E, and lane F shows the undigested pLPLσ. However, we saw no bands in any of the pControl lanes (Fig. 3B). This was likely because in between the first and the second run, we increased the plasmid yield of pLR-GFP and the pLPLσ, but not of pControl, due to time constraints. Therefore, the pControl DNA concentration was still very low when the second gel was loaded, which might have caused these bands to be too faint to be clearly seen. Moreover, lane C, for pLR-GFP, and lane F, for pLPLσ, showed smeared bands (Fig. 3B). This may be due to overloading of the sample, or due to the different shapes that undigested plasmids take, which results in various bands of DNA in the gel corresponding to the different rate the plasmid of the plasmids moving through the gel.

Ultimately, there were instances where the enzyme digestion cut the plasmid into the pieces we expected to see. These results, however, are inconsistent, as we did not see the same results for pControl and pLPLσ across both gels.

Sanger Sequencing and confirmation of pLPLσ. Along with performing restriction digest analysis, we sent pLR-GFP, pLPLσ and pControl for Sanger sequencing to confirm their identity.

After conducting Sanger sequencing, plasmid identity was successfully confirmed for pLPLσ, as sequencing results showed nearly complete alignment in close proximity to the M13 Reverse primer, confirming the identity of the plasmid as pLPLσ. On the other hand, the sequencing results for pControl showed a successful primer binding, but it was not in close proximity to the designed primer's binding location, and the DNA sequence received from GeneWiz does not match the original plasmid map of pControl. It is therefore possible that the plasmid does not match the map that accompanied it. However, the restriction enzyme digest indicated that the plasmid was cut into pieces coherent with pControl. The restriction enzyme digest shows that the plasmid we received has at least several similarities to the pControl used by Gaida et al. In the case of pLR-GFP, the sequencing process failed, stating that there was no primer-plasmid interaction in the sequencing process. This indicates that either the plasmid itself was absent in the reaction or the plasmid does not match the corresponding map. If the plasmid was present and it simply does not match the provided map, this would again seem to contradict the enzyme digestion process, as in the gel, we observed bands of DNA where they were expected following the digestion process. This suggests that the plasmid is at least similar to the pLR-GFP plasmid map. Additionally, given pLR-GFP's behavior in ccdB resistant and susceptible genes, it seems that the plasmid in question does contain *ccdB*, as indicated by the plasmid map for pLR-GFP, as well as the antibiotic resistance genes. Therefore we were not able to confirm the identity of neither pControl nor pLR-GFP. Two possible explanations for these results is that we might have received the incorrect plasmids or the incorrect reference sequence from the researchers at the University of Delaware. However, given the evidence from our restriction enzyme digest and the behaviour of pLR-GFP, we believe these plasmids to at least be similar to the plasmid maps provided. In the procedure by Gaida et al., the plasmids went through several steps of manipulation, where genes were inserted several times into the plasmid backbone. It is

possible that the plasmids obtained are from a different stage of their experiment than what the plasmid maps illustrated, which is why the plasmids pLR-GFP and pControl were similar in some ways to the plasmid maps, but different in their sequence. More investigation is required to confirm the exact identity of these two plasmids.

Limitations The methods outlined in this paper produced very low plasmid yields which in turn yielded faint bands in the restriction digest analysis. These low plasmid yields also did not meet the minimum concentration requirements for Sanger sequencing. Due to time limitations in this experiment, we were not able to fully establish the two-plasmid systems to drive heterologous promoter expression in *E. coli* using heterologous sigma factors. Other limitations include cost and access to the Invitrogen Gateway Cloning Recombination Technique. Reagents in this kit are relatively expensive and may be difficult to gain access to.

Conclusions In this paper, we demonstrate the initial steps towards establishing the two-plasmid system designed by Gaida et al. to increase heterologous promoter expression in *Escherichia coli,* using *Lactobacillus plantarum* sigma factor RpoD. Upon analyzing the plasmid maps of pControl, pLPLσ, pLR-GFP, and pUC-LR-GFP, we identify their roles in the two-plasmid systems. pLPLσ is an expression vector for *L. plantarum* RpoD. pControl functions as a negative control, with near identical construction as pLPLσ but containing a promoterless *gus* gene in the place of *rpoD*. pLR-GFP is the destination vector containing a promoterless GFP trap. Heterologous promoters are cloned into pLR-GFP via the Invitrogen gateway technique to establish an expression vector containing the heterologous promoter-GFP trap segment. Lastly, pUC-LR-GFP functions as the positive control, with near identical construction as pLR-GFP, but containing an additional lac promoter upstream of the promoterless-GFP segment. Upon insertion of heterologous promoters into pUC-LR-GFP, also via the Gateway technique, promoter transcription and GFP expression can be induced using IPTG despite the absence of RpoD, thus validating the experimental design. We then successfully propagate pControl and pLPLσ in *E. coli* DH5α, and pLR-GFP in *E. coli* JM109*,* the latter of which is a *ccdB*-resistant strain. Finally, we gained insight into the assembly and identity of pControl, pLPLσ, and pLR-GFP by restriction digest analysis, and confirmed the identity of pLPLσ via Sanger sequencing.

Future Directions Should future studies directly continue our research efforts, the identity of pControl, and pLR-GFP should be confirmed by repeating the Sanger sequencing process. It is recommended to perform another extraction of these plasmids, and to design new primers for each using the provided plasmid maps to verify if the plasmid maps indeed match the plasmids provided.

The Invitrogen Gateway technique can then be used to clone heterologous promoters into the pLR-GFP destination vector. Following this, co-transformation of pLR-GFP and pLPLσ into *E. coli* should proceed, along with GFP expression analysis using flow cytometry (Fig. 2.)

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