Undergraduate Journal of Experimental Microbiology and Immunology (UJEMI) Vol. 27:1-10

An assay for screening an *E. coli* green fluorescence protein promoter library

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SUMMARY Biosensors are valuable tools for understanding metabolic pathways and detecting organic molecules, particularly through the use of metabolite-sensing promoters. An important step in the development of biosensors is to create a reliable assay to screen for promoters that respond to metabolites of interest. Although previous studies have explored assay and biosensor development, replicability and feasibility within our current laboratory has yet to be determined. In this study, we aimed to perform the preliminary steps in developing an assay for screening of an *E. coli* promoter library by using L-phenylalanine as a model substrate. We obtained a green fluorescence protein promoter library containing 1,820 different promoter regions of *E. coli* K12 strain MG1655. We performed a pilot study with a subset of the library representing 96 clones to simplify our early stage experiments. Promoter activity was measured as green fluorescence. Heat stress was used as a control condition to assess promoter responsiveness. Serial dilutions of green fluorescence existing clones were performed to test the dynamic range of the assay. Subsequent screens using phenylalanine were done to find promoters responsive to this metabolite. In our subset of 96 clones, we were able to identify five strains that showed altered GFP expression in response to the addition of phenylalanine. Our work here establishes a system to carry out fluorescence-based assays to screen an *E. coli* promoter library.

INTRODUCTION

Transcription-based biosensors are tools for understanding bioprocesses at a single-cell resolution, demonstrating broad applicability in areas such as medicine, pharmaceuticals, food and process control, and most popularly in medical diagnostics (1). For biosensor design, metabolite sensing proteins are often utilized, which are activated upon the binding of an effector molecule. When effector molecules bind to corresponding metabolite sensing proteins, gene expression can be controlled using a measurable output signal such as fluorescent reporters which results from the interaction of the two molecules (2). Identification of appropriate biosensor candidates is hindered by the abundance of targeted promoters, demanding a technique for rapid detection of promoters for sensor design (3).

One approach to identify DNA sequences suitable for biosensor design is to screen libraries where promoters have been linked to genes encoding detectable products like green fluorescence protein. Here we carry out preliminary steps to develop an assay based on GFP fluorescence to screen for metabolite-sensing promoters.

An effective design of high-throughput screening assays considers the signal responses in regards to influential factors such as a change in environmental stimuli (4). Such stimuli could consist of environmental factors, like temperature or extracellular metabolites. Additionally, whether a stimulus is dependent only on the compounds being tested and the duration it takes for the response to occur are also essential factors to consider when developing an assay (4). Considering these parameters, the basis of an effective high-throughput screening design can be defined.

In this study, we screened an *E. coli* promoter library which was created by Zaslaver et al. (5). The Alon library, named after Alon Zaslaver, consists of 1,820 different promoter regions which represents a minimum of 75% of the promoters found in *E. coli* (5). The promoters were cloned into the cloning site upstream of *gfpmut2* and therefore controlled transcription of *gfpmut2* (5). The *E. coli* promoter-*gfpmut2* fusions allow for the expression of GFP, which is required to obtain accurate measurements of promoter activity (5). Previous

Published Online: September 2022

Citation: Joean Lu, Daniel Mun, Andrew Song, Catherine Yu. 2022. An assay for screening an *E. coli* green fluorescence protein promoter library. UJEMI 27:1-10

Editor: Andy An and Gara Dexter, University of British Columbia

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studies have shown that this library responds to diauxic shift, where cells were grown on a medium with limited glucose and abundant lactose (5). Additional stress genes that are found in *E. coli* include the heat shock promoters that have increased activity due to a temperature change from 37°C to 42°C (6). In the current study, heat stress is employed to monitor a single parameter, GFP expression, in response to varying temperatures to imitate the effects of environmental stimuli.

Additionally, we use L-phenylalanine as a model substrate to screen for inducible promoters that are responsive to environmental metabolites. Previous studies have successfully used this library for the identification of L-phenylalanine-responsive promoters (3). Phenylalanine is an amino acid that plays an essential role as a precursor for artificial sweeteners, taste and aroma enhancers, and as a building block for pharmaceutical products such as infusion fluids and HIV protease inhibitors (8, 9). With the increasing global demand of this metabolite, production of phenylalanine through microbial fermentation using genetically-engineered strains has become a dominating process (10, 11). *E. coli* is an important organism in producing L-phenylalanine via the shikimate pathway as microbial fermentation is dominant in its production (3, 10). Thus, the development of a biosensor for phenylalanine-responsive strains may lead to the discovery of microbes with increased phenylalanine production (3). The current study aims to reproduce the results of Mahr et al. to authenticate its reproducibility and replicate experiments to find trends and patterns in the results.

To this end, our experiment is interested in developing a fluorescence based-assay that can be used to identify promoters that respond to an induction substrate. A positive correlation between GFP expression and concentration of phenylalanine was observed. In addition, five strains were identified to have altered GFP expression in the presence of phenylalanine.

METHODS AND MATERIALS

Bacterial strains, media and growth conditions. The *E. coli* strain MG1655 library of promoters fused to *gfpmut2* was developed by Zaslaver et al. and provided as a gift from the Hallam Lab at UBC (5). We obtained a 384 well plate of the Alon library and divided it into 4x96 well plates. This study utilizes the first 96-well plate of the given library. Clones are named after the wells on the 96-well plate. The *E. coli* library was obtained from -80°C storage and pre-cultivated in Luria Bertani medium for 8 h at 37°C and 170 rpm (12). 2µL of this cell culture was inoculated into wells of a microtiter plate containing 200µL of M9 minimal media (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mL/L trace element solution [3 µM ammonium molybdate, 400 µM H_3BO_3, 30 µM $CoCl_3$, 10 µM $CuSO_4$, 800 µM $MnCl_2$, and 10 µM $ZnSO_4$; filter sterilized]) and 50 µg/mL kanamycin, grown overnight at 37 °C and stored at 4°C until needed. Media stocks were stored at room temperature.

Microplate cultivation. Cell growth curves were performed in 96-well microtiter plates using the BioTek Epoch 2 Microplate Spectrophotometer. In the BioTek instrument, *E. coli* cells were incubated at 37 °C with an agitation frequency of 425 cpm (3mm). OD_{600} readings were measured every 10 minutes over a 16-hour period. 96-well microtiter plates were inoculated with 2µL of *E. coli* pre-culture and 200µL of M9 minimal media. GFP fluorescence was measured at an excitation of 485 nm and emission of 528 nm using the Tecan SpectraMax Gemini EM microplate reader.

Measurement of growth curves. For comparison of bacterial growth in the presence of an induction substrate, the mean of triplicate readings was plotted every 0.5 hours to obtain the standard growth curve at different phenylalanine concentrations. To determine the relationship between GFP expression and cell growth, stationary-phase synchronized *E. coli* MG1655 suspension was serially diluted (1:2, 1:4, 1:8) using fresh M9 minimal media. To determine the response of *E. coli* cells under environmental stress, control plates were incubated at 37°C and then heat stressed at 42°C for 16 hours.

Statistical analysis. All statistical analysis was performed using Microsoft Excel. Values were reported as mean value ± standard deviation. Fold induction was calculated as the ratio of GFP fluorescence and OD_{600} values.

RESULTS

GFP expression of promoters are responsive when shifted from growth at 37°C to growth at 42°C.

To determine whether the *E. coli* K12 library contained promoters that respond to environmental stimuli, GFP expression was measured following heat stress using microplate reader-based methods described above. The 96 strains from plate 1 of the Alon library were selected and replicated into two plates. In parallel, the two plates of identical clones were incubated at 37°C and 42°C for 16 hours. Both an increase and decrease of GFP expression were observed in response to the heat stress at 42°C relative to incubation at 37°C. The raw data was normalized prior to analysis by determining the GFP/OD_{600} ratio in both treatment groups. Up to 1.84 fold increases of GFP expressions were observed, while 0.37 fold decreases were observed in response to the heat stress (Fig.1). Figure 1 includes the clones that showed both the greatest and smallest differences due to the shift in temperature and are representative of all the clones that were tested. This result validated the *E. coli* library for GFP expression by screening for heat responsive promoters. The results suggest that GFP expression of promoters found in the *E. coli* K12 library are temperature dependent and that the library contains promoters that are responsive to environmental stimuli.

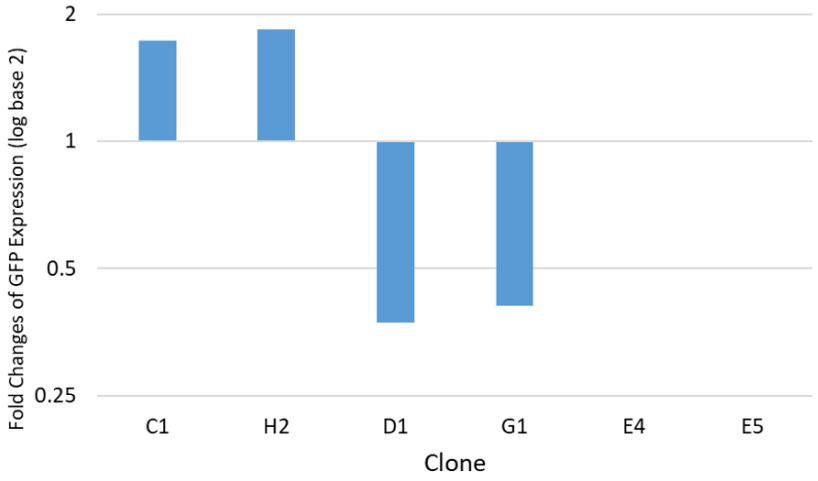

FIG. 1 Fold induction graph of GFP expression following heat stress at 42°C. Duplicates of plate 1 of the Alon library were incubated at 37°C and 42°C in parallel for 16 hours. GFP expression values were divided by OD_{600} for normalization of data for each condition. Fold induction is given as a ratio of fluorescence expressed under treatment conditions at 42°C to conditions at 37°C.

GFP expression decreases linearly with OD_{600}.

To determine whether there is a proportional relationship between GFP expression and concentration of cells, three clones H6, F10 and E2, were randomly selected from the 96 strains from plate 1 of the Alon library. This experiment acted as a control to further verify the linear relationship between fluorescence expression and cell concentration, allowing us to attribute future changes in GFP expression to tested experimental conditions (i.e. phenylalanine) and not to changes in concentration. Each clone was inoculated in duplicate in the same 96-well plate and incubated at 37°C for 16 hours overnight using the microplate reader-based methods described above. After 16 hours, GFP and OD_{600} readings of the undiluted clones were measured as a control to represent basal levels of fluorescence expression and cell growth before dilution. 100µL of the cultures was diluted in another 100µL of M9 minimal media to make a 1:2 dilution, and both GFP and OD_{600} readings were taken. This process was repeated to make 1:4 and 1:8 dilutions. It was found that a decrease in concentration of cells (OD_{600}) led to a corresponding decrease in GFP expression (Supplemental Fig. 1). However, at OD_{600} concentrations of less than 0.04, there was no longer a proportional relationship seen between GFP and OD_{600}. This resulted in unreliable GFP readings at these low OD_{600} concentrations. Therefore, a range for GFP expression measurements was determined to be OD_{600} readings greater than 0.04 (Supplemental Fig. 1).

This minimum OD_{600} value was taken into consideration when analyzing the following sections of this study.

Figure 2 shows the change in GFP:OD_{600} ratio values from a 1:1 dilution to a 1:2 dilution. This ratio normalized fluorescence and OD_{600} values (Fig. 2). Theoretically, the GFP:OD_{600} ratio for each clone should not change significantly because changes in concentration lead to corresponding changes in GFP expression (Fig. 2). For clones F10 and E2, the lack of significant difference in ratios between dilutions suggest that changes in concentration alone were not responsible for changes in GFP expression. However, clone H6 showed significant change in ratio values even after normalization, suggesting that differences in GFP expression were due to sources of error (Fig. 2).

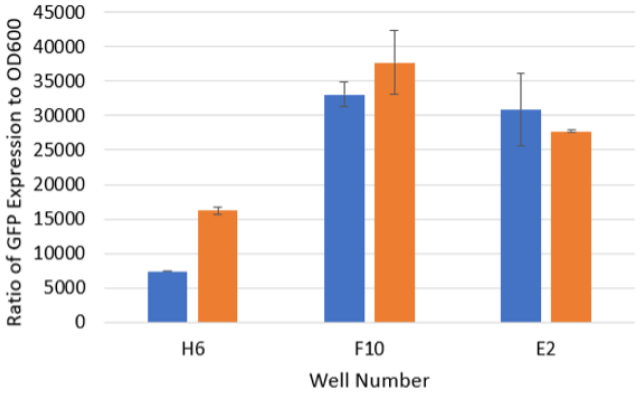

FIG. 2 Effect of cell concentration on the ratio of GFP to optical density. GFP expression values were divided by OD_{600} for normalization of data for each condition. Blue bars represent 1:1 undiluted samples, and orange bars represent 1:2 dilutions. Error bars represent standard deviation.

E. coli cell growth increased in the presence of phenylalanine

Next, we aimed to determine the effect of varying concentrations of phenylalanine on *E. coli* cell growth. The four clones H6, F10, E2 and D5 were incubated in M9 minimal media and treated with either 0mM, 1mM, 3mM, or 5mM phenylalanine at the start of the 16 hour incubation period. We analyzed the effects of phenylalanine on cell growth using microplate reader-based methods described above. The control condition utilizing 0mM phenylalanine represents basal levels of cell growth and provides a standard of comparison for treatment conditions. The treatment conditions were performed in triplicates.

The growth yield for clone H6 increased with the increasing concentration of phenylalanine from 0mM to 5mM (Supplemental Fig. 2). The growth yield for clone F10 increased with the increasing concentration of phenylalanine from 0mM to 5mM (Supplemental Fig. 3). For clone E2, the growth yield was higher at 1mM, 3mM and 5mM phenylalanine compared to control conditions (Supplemental Fig. 4). The growth yield for clone D5 increased with the increasing concentration of phenylalanine from 0mM to 5mM (Supplemental Fig. 5). For all clones, the treatment conditions had higher growth rates compared to the control condition and the greatest growth rate was observed at 1mM phenylalanine (Supplemental Fig. 2-5). These results show that with the addition of phenylalanine, *E.coli* cell growth increases.

Optimal concentration for screening determined to be 3mM phenylalanine.

In order to study the effect that varying concentrations of phenylalanine had on GFP expression, four clones H6, F10, E2 and D5, were incubated in M9 minimal media and treated with either 0mM, 1mM, 3mM, or 5mM phenylalanine at the start of the 16 hour incubation period. We compared and quantified the effects of phenylalanine on GFP expression using microplate reader-based methods described above. The control condition utilizing 0mM phenylalanine represents basal levels of GFP expression and provides a standard of comparison for treatment conditions. Fold induction of GFP expression under each treatment condition was obtained by normalizing fluorescence values by OD_{600} readings and taking the ratio of GFP expression in treatment conditions over GFP expression in the control condition. The treatment conditions were performed in triplicates. Fold induction under each treatment condition was plotted in Figure 3.

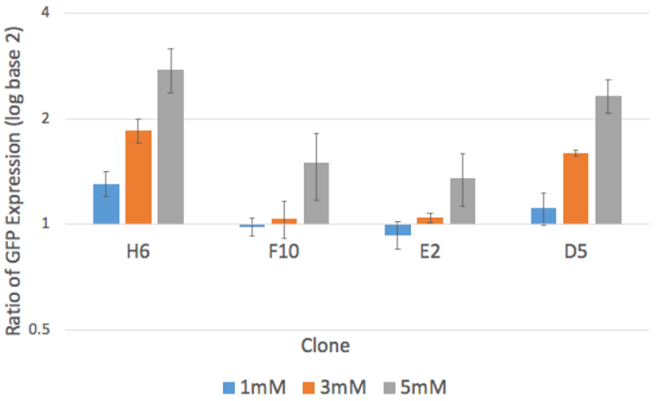

FIG. 3 Fold induction of GFP of clones H6, F10, E2 and D5 in the presence of varying concentrations of phenylalanine. Strains were cultured for 16 h in M9 minimal media with 1mM, 3mM, or 5mM phenylalanine. Control conditions were cultured with 0mM phenylalanine. Blue bars represent 1mM phenylalanine treatment, orange bars represent 3mM phenylalanine treatment, and gray bars represent 5mM phenylalanine treatment. Fold induction is given as a ratio of fluorescence expressed under treatment conditions to control conditions. GFP expression values were divided by OD_{600} for normalization of data for each condition. Experiments were performed in triplicate. Error bars represent standard deviation.

The results show that GFP expression increases with phenylalanine concentration (Fig. 3). 5mM phenylalanine had the greatest effect on GFP expression with average fold change reaching 1.99-fold higher in the 5mM treatment, followed by 1.38-fold in the 3mM treatment and 1.08-fold in the 1mM treatment. 1mM and 3mM phenylalanine had lesser effects on GFP expression with 1mM treatment having the least effect across all four clones tested (Fig. 3). By the growth curves and fluorescence-based assay, we determined that 3mM was the optimal concentration to perform further screenings as it did not severely impact cell growth as seen in the 5mM treatment but still showed significant modulations on GFP expression, unlike the 1mM treatment (Fig. 3, Supplemental Fig. 2-5). This is consistent with the concentration of phenylalanine used in the screening of the same promoter library by Mahr et al. which was performed using 3mM L-alanyl-L-phenylalanine as the effector molecule (3).

In addition to a positive correlation between fluorescence induction and phenylalanine concentration, the level of induction in response to all tested concentrations of phenylalanine was observed to be consistent in each individual clone (Fig. 3). The fold change in fluorescence expression was higher in response to all phenylalanine concentrations in clone H6 than it was in clones F10, E2, or D5 (Fig. 3). Similarly, the fluorescence expression was the lowest in clone E2 compared to the other clones tested (Fig. 3). These results indicate that levels of fold induction differ between clones.

Screening of the reporter library yielded one clone with increased GFP in response to phenylalanine.

We asked if the current fluorescence-based assay would be able to identify clones that responded to phenylalanine. Each of the 96 clones were incubated in M9 minimal media and treated with 3mM phenylalanine at the start of the 16 hour incubation period. Microplate reader-based methods were used to compare and quantify changes in GFP expression in the presence of phenylalanine. The control condition was incubated in M9 minimal media with no phenylalanine and measured using the same protocol. The control condition represents basal levels of GFP expression and provides a standard of comparison for treatment conditions. Fold induction of GFP expression for each clone was obtained by normalizing fluorescence values by OD_{600} readings and taking the ratio of GFP expression in treatment conditions over GFP expression in the control condition. The treatment condition was performed in triplicates. Fold induction of GFP expression for five clones of interest was plotted in Figure 4.

By using the fluorescence assay that is currently in development, we observed that fold induction in GFP expression may increase or decrease in response to phenylalanine treatment (Fig. 4). The five clones plotted in Figure 4 demonstrated the greatest changes in GFP expression out of the 96 clones that were tested. The majority of strains did not demonstrate changes in fluorescence expression with fold induction and remained around 1.0-fold (Supplemental Fig. 6). Clone E5 had the greatest change in fluorescence and was the only clone that was determined to be statistically significant when compared to all 96 clones that were tested (Supplemental Fig. 6). These results indicate that changes in fluorescence fold induction differs between individual strains of the tested promoter library. These findings also

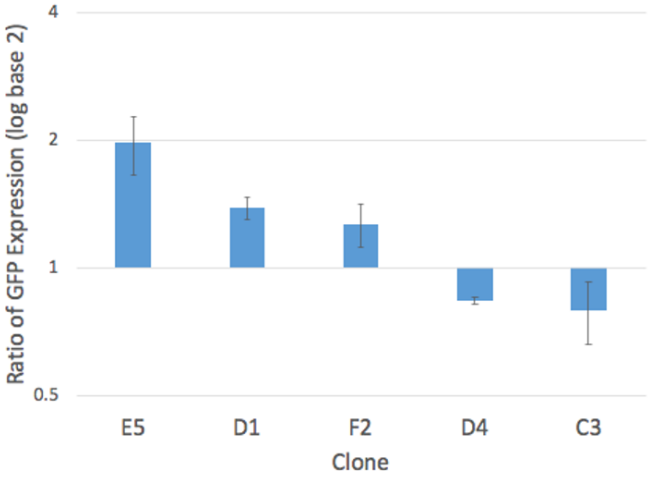

FIG. 4 Fold induction of GFP in 5 identified *E. coli* K12 strains with the greatest change in fluorescence in the presence of 3mM phenylalanine. Strains were cultured for 16 h in M9 minimal media with 3mM phenylalanine. Control conditions were cultured with 0mM phenylalanine. Fold induction is given as a ratio of fluorescence expressed under treatment conditions to control conditions. GFP expression values were divided by OD600 for normalization of data for each condition. Experiments were performed in triplicate. Error bars represent standard deviation.

indicate that the fluorescence-based assay in development can measure modulations in GFP expression in response to phenylalanine.

DISCUSSION

Here, we describe preliminary steps to develop a fluorescence-based assay by screening a subsection of a GFP promoter-trap library and its use as a tool to study and compare the diverse regulatory responses of *E. coli* promoters in reporter gene libraries. Effective design of high-throughput screening assays takes the nature of response to environmental stimuli into consideration (13). The steps we took to establish this fluorescence-based assay are outlined in a flowchart that describes the workflow followed in our study (Fig. 5). First, we obtained the Alon promoter library and incubated 384 clones in M9 minimal media overnight to generate 4x96 well working plates with liquid cultures. We used the working plates to inoculate fresh media and generate cultures in further screenings. After obtaining the working plates, we wanted to assess basal levels of fluorescence expression by testing the effect of heat stress on GFP induction. Next, we tested the effects of phenylalanine on growth and identified 3mM phenylalanine to have the least effect on growth while maintaining fluorescence induction potential. We also established an OD_{600} range which shows proportional GFP expression. Finally, we tested the effect of 3mM phenylalanine on GFP expression and identified 1 clone that showed significant modulation in response to phenylalanine.

Cell concentration has minimal effect on GFP expression.

For all screenings in this study involving fluorescence measurements and GFP fold induction, we used cell concentration (OD_{600}) to normalize the GFP expression readings of each clone. The GFP:OD_{600} ratio helped control for sample-to-sample variability in GFP caused by a wide range of OD_{600} values (15). Differing OD_{600} values can result in altered GFP values as fluorescence becomes a measure of cell concentration rather than promoter activity. The GFP:OD_{600} ratio also confirmed a range of OD_{600} values for which fluorescence was proportional to the cell concentration. A linear relationship between GFP and concentration was expected (16), as supported by our data (Supplemental Fig. 6-7). The 1:4 and 1:8 dilutions (OD_{600} 0.04-0.045) demonstrated similar fluorescence and OD_{600} values and did not follow a linear relationship as seen with the 1:1 and 1:2 dilutions (Supplemental Fig. 7). This suggests that when cell concentrations are too low, GFP expression measurements are not reliable and their accuracy and usefulness in our study is compromised. We suspect that this may be due to the microplate reader being unable to accurately measure OD_{600} values below 0.04, as low cell concentrations fall below the threshold of detection on the reader. Low cell concentrations can also cause GFP levels to fall below the detection limit on the fluorescence reader. Thus, in our normalization control (Fig. 2), only the 1:1 and 1:2 dilutions were used as they were within the range of data that was considered to be reliable. As expected, the GFP: OD_{600} ratios for clones F10 and E2 showed very little change after dilution, confirming that they correct for changes in concentration (Fig. 2). Lastly, the ratios help us confirm that changes in OD_{600}

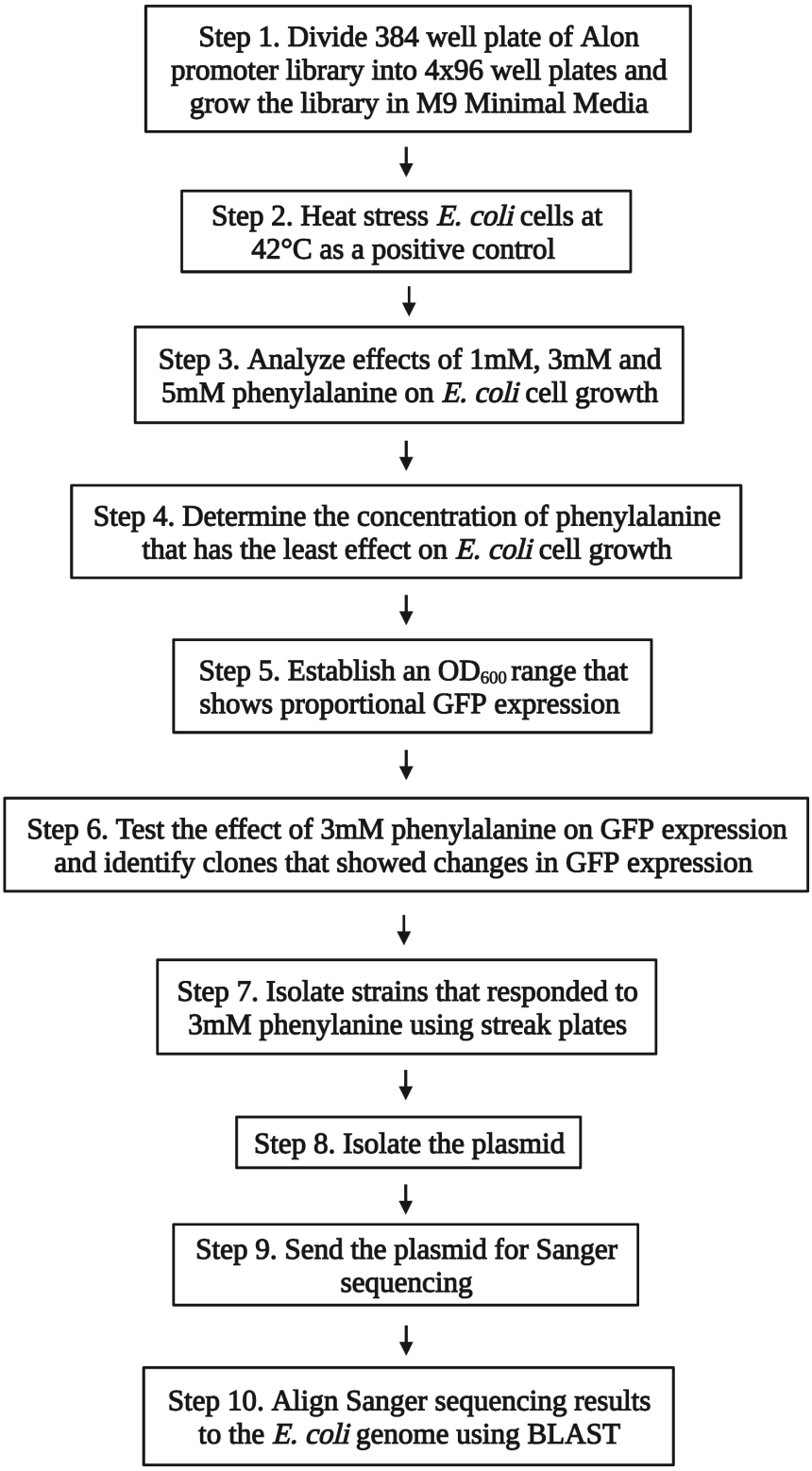

FIG. 5 Flow chart depicting the process of developing an assay to screen an *E. coli* promoter library. The Alon promoter library was obtained as a gift from the Hallam Lab. Phenylalanine was used as a model substrate.

alone do not account for changes in GFP expression, allowing us to attribute future changes in GFP to experimental conditions, not concentration of cells.

Fluorescence-based screen using phenylalanine.

The analysis of GFP fold induction of clones H6, F10, E2, and D5 in response to different concentrations of phenylalanine revealed that each clone responded to phenylalanine in an individual manner. Clone H6 demonstrated the highest fluorescence fold-change, regardless of phenylalanine concentration tested. This is a positive sign that the assay is actually reflecting the properties of each clone as it indicates that each strain in the library is unique and will respond to effector molecules differently depending on the promoter it contains. A positive correlation between GFP expression and concentration of phenylalanine was also observed during this screening. Even after normalizing GFP expression by the OD_{600} readings, treatment with higher phenylalanine concentrations still resulted in greater fluorescence induction. Future studies could assess the effects of higher concentrations of phenylalanine on GFP expression and determine if this pattern remains consistent with increasing levels of phenylalanine. Phenylalanine catabolism in *E. coli* is not well documented in literature. Therefore, gaining an understanding of why increased levels of phenylalanine result in greater fluorescence can contribute to the current dearth of knowledge on the role of amino acids in gene expression.

During screening for phenylalanine-responsive promoters using the developed fluorescence-based assay, five clones were identified based on significant changes in GFP expression (Fig. 4). Three of the clones exhibited upregulation in fluorescence while two of the clones demonstrated lower fluorescence expression in response to phenylalanine (Fig. 4). However, only one of the clones were statistically significant when compared to the fold induction and standard deviations of all 96 clones (Supplemental Fig. 6).

Our screening showed that the highest fold-change in response to phenylalanine was only a 1.94-fold increase while the majority of promoters in the tested library did not exhibit any significant fold-change (Fig. 4, Supplemental Fig. 6). This is expected as we focused our preliminary assay development on a small subsection of the promoter library that only screened 96 out of 1,820 different reporter strains. This fluorescence-based assay could be further developed and scaled up to screen the entire library. Further screening may identify clones that respond with a greater fold-change in fluorescence and comparatively refute the consideration of clones identified in this screening as phenylalanine-responsive strains.

Limitations One limitation of this study is the low levels of cell viability and growth of *E. coli* clones in M9 minimal media. M9 minimal media was used for its low auto-fluorescence and minimal interference with GFP readings (3). However, the OD_{600} values obtained were consistently low and occasionally resulted in no growth of the *E. coli* strains in the library. As previously mentioned, low OD_{600} values can be a major limitation as values that fall below the threshold of detection will result in inaccurate readings of both cell concentration and fluorescence expression. Use of different carbon sources and possibly different minimal media is suggested for future studies. Depending on the strains of *E. coli* and carbon source, M9 minimal media may not be recommended (18). Alternative media such as LeMaster-Richards (LMR) or Studier phosphate buffer (SPG) may be suggested for optimal growth (18). The low OD_{600} values may have also been due to the limited maximal culture volume associated with microtiter plates which resulted in poor aeration. Another limitation may be the use of manual inoculation methods to transfer media and cell cultures. These methods may have influenced the inconsistent starting OD_{600} values observed in this study. Automated systems or the use of multi-channel pipettes may be more accurate and consistent. This would have allowed for a standard initial OD_{600} value to be set for each experiment and for the entire library to be screened more efficiently.

Furthermore, another limitation to the study was the lack of a strong positive control for substrate-induced fluorescence induction. Since none of the strains in the Alon library had been previously identified or labeled, known substrate-responsive promoters could not be tested as a positive control. Instead, we performed heat-stress experiments as a proof-of-concept screen to confirm that the library responded to environmental stimuli. However, known metabolite-sensing promoters were not tested so we did not have the ideal comparison

for fluorescence values obtained during our screening. Lack of this comparison may reduce the reliability of the presented results. Other metabolites, such as glucose or galactose, can be used as the effector molecule to identify other substrate-responsive promoters in the library. Once these promoters have been identified through sequencing, they can be used as a stronger positive control for future screenings.

Conclusions As stated in our research question, this study presents preliminary steps taken to develop a fluorescence-based assay that can be used to assess substrate-induced promoter activity in *E. coli*. Steps 1-6 of the flow chart was completed, including testing the effects of heat stress on GFP induction, assessing the effects of phenylalanine on cell growth, determining the optimal phenylalanine screening concentration and its effect on fluorescence, establishing a working OD_{600} range which shows proportional GFP expression, and identifying clones that showed changes in GFP expression during the final screening (Fig. 5). Five clones out of a subset of 96 were identified to have altered GFP expression while screening using phenylalanine as a model substrate. Steps 7-10 of the flow chart, including plasmid isolation and sequencing to characterize specific promoters could not be completed (Fig. 5).

Future Directions Using the fluorescence-based assay, five clones were identified based on changes in GFP in response to phenylalanine. In order to characterize these clones, strains that responded to phenylalanine will be isolated using streak plates. The next step is to isolate the plasmids and send samples for Sanger sequencing to confirm the nucleotide sequence of the isolated strains. After aligning sequencing results to the *E. coli* genome using BLAST, the identity of the isolated promoters will be revealed which will provide a strong indication on the robustness of the assay at hand.

Continuing with the example of using phenylalanine as the model substrate, other aromatic amino acids such as tryptophan and tyrosine should be tested as effector molecules to assess the responsiveness of the current assay. Screening with tryptophan and tyrosine will provide greater insight on the specificity of the assay as it may reveal whether fluorescence was indeed induced by phenylalanine or by aromatic amino acids in general. If fluorescence is significantly increased in the presence of phenylalanine but not tryptophan or tyrosine, this would provide greater evidence that the promoter is specific to phenylalanine. The *mtr* promoter is known to be regulated by a tryptophan repressor (TrpR) and a tyrosine regulator (TyrR) (3, 17). Similarly, if increased fluorescence was detected in the presence of tyrosine and phenylalanine and decreased fluorescence was detected in the presence of tryptophan, this would strongly support the verification of the *mtr* promoter. The *mtr* promoter was previously found to be enriched in a high-throughput screening of the Alon library using fluorescence-activated cell sorting (FACS) by Mahr et al (3). Identification of this promoter is important because it would confirm the rigor of the assay in measuring induction of fluorescence.

In terms of future experiments that could be done to improve the accuracy and reliability of this assay, further optimizations around media, growth conditions, and GFP measurements should be performed. One of the major limitations of this study was the low OD_{600} values obtained from all cultures grown in M9 minimal media. The OD_{600} values obtained in this study peaked around 0.200, typically remaining around 0.090-0.100. Future studies could test the effects of different types of media on cell growth and auto-fluorescence to avoid issues associated with low OD_{600} values and compounds precipitating out of solution. Further adjustments on growth conditions, such as increasing or decreasing the shaking frequency of the microplate reader, the length of incubation, and the timing of fluorescence readings can be optimized to determine the prime conditions for cell growth in microplate readers.

The fluorescence-based assay used during this study can also be scaled up to screen the rest of the Alon library or applied to other *E. coli* promoter libraries. Only 96 out of 1,820 reporter strains of the Alon library were screened in this series of small-scale experiments. Our screening with phenylalanine showed that the highest recorded fold-change in fluorescence was only a 1.97-fold increase while the majority of promoters in the tested library did not exhibit any significant fold-change (Supplemental Fig. 6-7). Hence, next steps

could include using the novel fluorescence-based assay to screen a larger portion of the Alon library and to identify strains that respond with a greater fold-change in fluorescence.

Finally, the promoters identified using this fluorescence-based assay may have important implications in the development of transcription-factor based biosensors that detect extracellular effector molecules (3). Biosensors are valuable tools for the identification of novel metabolite producers and the development of more efficient microbial production platforms. The preliminary steps taken in this study aimed to establish a strong foundation for the further development of a robust fluorescence-based assay that allows for rapid detection of promoters for biosensor design.

ACKNOWLEDGEMENTS

We would like to acknowledge D. Oliver, C. Haney, D. Yanez Ortuno, J. Muileboom, A. Noonan for assistance and discussions, and funding from the UBC Department of Microbiology and Immunology.

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