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Advancement of Oxygen Biosensor in Escherichia coli

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Abstract

Microbial biosensors can be used to provide information about the cells' environment in large-scale fermentations. In this project an oxygen sensitive biosensor is being developed in *Escherichia coli* to determine what kind of conditions cells are growing in: aerobic or anaerobic. This project specifically studies expression from the fumarate and nitrate reductase (FNR) promoter (P_{FNR}) under aerobic and anaerobic conditions. In fluorescence experiments, the expression levels of green fluorescent protein (GFP) were used to quantify the effectiveness of the P_{FNR} in the DH5α, MG1655 and BL21 strains of *E. coli*. The negative control of the experiments, which are the *E. coli* strains without a GFP plasmid, showed very high levels of background fluorescence until the emission and excitation wavelengths of the fluorescence plate reader were adjusted. Overall, the P_{FNR} expressed more GFP under anaerobic conditions in the DH5α and BL21 strains. The results of the MG1655 strain indicated that the strain has the *fnr*- genotype and did not draw any definite conclusions of P_{FNR}. Another oxygen sensitive promoter plasmid, pKVS-*vgb*-GFP, was constructed using circular polymerase extension cloning (CPEC) to be tested in the future. A positive control plasmid, pKVS-J23101-GFP, was also constructed using CPEC that should not produce as high of an amount of GFP relative to the current positive control plasmid. Once verified as the correct plasmid size, the new positive control can be used in future fluorescence experiments.

Acknowledgements

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Introduction

The fermentation process employs the ability of microorganisms to break down materials and produce a desired product such as antibiotics, foods and biofuels. However, when the fermentation process is scaled-up, non-homogenous conditions are often created, which lead to side product formation and a lower product yield. [1] Components such as oxygen, pH and substrates can be distributed unevenly, so microbial biosensors are used to detect what type of conditions the microorganisms are growing under. These experiments analyze the development of an oxygen sensitive biosensor in several strains of *E. coli* that utilize the P_{FNR}.

E. coli use the FNR protein to switch between aerobic and anaerobic respiration. [2] The FNR protein is composed of an iron-sulfur cluster that can be oxidized or reduced. Under anaerobic conditions the cluster forms a dimer, which is much more effective at binding to DNA so that transcription can occur and genes can be expressed. Then, under aerobic conditions the FNR protein transcription factor breaks apart into the monomer form and becomes less efficient at binding to DNA. [3] The environment's oxygen levels control whether not the FNR protein binds to the P_{FNR} promoter region and the desired gene is expressed. [4] The FNR protein is used in a variety of applications to detect oxygen or dissolved oxygen levels that cells are growing in. [5] Also, the FNR protein can be used with other bacteria besides *E. coli* such as cyanobacteria like *Synechocystis* and still detect oxygen levels because of its ability to switch growth metabolism between anaerobic and aerobic conditions. [6]

Materials and Methods

Fluorescence Experiments

Fluorescent experiments were conducted in the DH5α, MG1655 and the BL21 strains of *E. coli* (Table 1). The FNR-GFP and pTrc-GFP plasmids in the DH5α strain were miniprepped using the New England BioLabs (NEB) Monarch Plasmid Miniprep Kit and the BL21 competent cells were transformed with the plasmids. The transformed cells were plated on Luria-Bertani-Ampicillin (LB-Amp) plates and incubated overnight at 37 °C. The plated colonies were then used to store the new strain in a 15% glycerol stock. Unlike the BL21 strain, the DH5α and MG1655 strains were already prepared by another research student, so those strains did not require an additional transformation.

Strain	Positive Control	Experimental	Negative Control
DH5α	pTrc-GFP in DH5α	FNR-GFP in DH5α	DH5α strain
MG1655	pTrc-GFP in MG1655	FNR-GFP in MG1655	MG1655 strain
BL21	pTrc-GFP in BL21	FNR-GFP in BL21	BL21 strain

Table 1. Three strains of *E. coli*, DH5α, MG1655 and BL21, were tested in the fluorescence experiments. Within each experiment for a strain there was a positive control, experimental and negative control. The positive control contained the pTrc-GFP plasmid in the desired strain, the experimental contained the FNR-GFP plasmid in the desired strain and the negative control was the desired strain without a GFP plasmid.

For each experiment of a particular strain, a liquid starter culture was made of the negative control that does not contain GFP, the positive control with the pTrc-GFP plasmid and the experimental strain with the FNR-GFP plasmid. All cultures contained 3 mL of LB medium and the corresponding colony. 3 µL of 100 mM ampicillin was added to the positive control and experimental cultures. All of the liquid cultures were incubated at 37 °C and 250 rotations per minute (rpm) overnight. Next, 300 µL of the starter culture was used to inoculate a flask of 30 mL of LB medium. 30 µL of 100 mM ampicillin were added to the flasks of the experimental and positive control and 30 µL of the 100 mM IPTG inducer was pipetted into the positive control flask. The flasks were plugged with foam and incubated at 37 °C and 250 rpm for 2 hours. Then, 1 mL samples were taken from each of the flasks and 15 mL of the culture in the flask were pipetted into a Hungate tube. The Hungate tubes were capped and provided the anaerobic conditions because there was very little head space in the tube once the culture was added. The cultures in the Hungate tubes and flasks continued to grow at 37 °C while 1 mL samples were taken at 2, 4 and 20 hours after the initial sample was taken. A pipet was used to remove the sample from the cultures growing aerobically in the flasks and a 1 mL syringe was used to take the sample from cultures growing micro-aerobically in the Hungate tubes.

Each 1 mL sample was centrifuged for 2 minutes at 13,000 rpm and the supernatant was discarded. Then, the cells were suspended in 0.5 mL of phosphate buffered saline (PBS) and this process was repeated a second time. At the 0 and 2 hour intervals the cells were resuspended in 0.5 mL of PBS and at the 4 and 20 hour intervals the cells were resuspended in 1 mL of PBS. Next, 200 µL of each sample were loaded into a well plate, so the fluorescence and absorbance readings could be taken. The fluorescence of each sample was measured on the Spectra Gemini plate reader on medium sensitivity at an excitation wavelength of 490 nm and an emission wavelength of 510 nm. The fluorescence of the second experiment with the DH5α strain was measured using an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Finally, the absorbance was measured on the BioTek plate reader at an absorption wavelength of 630 nm. The fluorescence measurement of each sample was divided by the corresponding absorbance value so that the fluorescence was normalized by number of cells present.

pKVS-J23101-GFP and pKVS-vgb-GFP Plasmid Construction and Transformations

DH5α competent cells were made using the standard protocol. The pKVS plasmid backbone and J23101-GFP construct were amplified and purified under the work of a previous research student. The purified DNA was used in a CPEC reaction, with an annealing temperature of 55 °C, to form one complete plasmid. Next, the DH5α competent cells were transformed with the CPEC product and plated on LB-Amp plates to grow at 37 °C overnight. The CPEC colonies that grew were amplified using the standard colony PCR method and checked using gel electrophoresis. A 0.7% agarose gel was run for 75 minutes at 82 volts to verify that the pKVS-J23101-GFP plasmid was accurate. Also, the CPEC colonies were used to make a 15% glycerol stock and to store the plasmid long-term. A double digest was done on the pKVS-J23101-GFP plasmid using the NEB CutSmart buffer, the XbaI restriction enzyme and the PstI–HF restriction enzyme.

The DNA of the pKVS backbone was also previously amplified as part of another student's work. The *vgb* promoter was amplified by colony PCR that used primers RK2 and RK3 (Attachment 1). The pTrc-GFP plasmid was prepared using the NEB Monarch Plasmid Miniprep Kit. Then, 0.5 µL of the miniprepped plasmid was used in a PCR reaction to amplify the GFP DNA. The *vgb* and GFP PCR products were

checked using gel electrophoresis. A 1.3% agarose gel was run at 82 volts for 75 minutes. Then, the PCR product of the *vgb* and GFP were cleaned with the NEB Monarch PCR and DNA Cleanup Kit. Elution buffer was used instead of TE buffer to bring the volume of the reaction up to the recommended minimum of 20 µL. Next, the pKVS backbone, *vgb*, and GFP PCR products were all connected to form one plasmid using CPEC with an annealing temperature of 55 °C. After the CPEC reaction, the product was digested with the DpnI enzyme. 2 µL of the enzyme was added to the CPEC product and incubated for 1.5 hours at 37 °C. Next, the digested DNA was heat inactivated by incubating the product at 65 °C for 20 minutes. The DH5α competent cells were then transformed with the digested CPEC product, plated on an LB-Amp agar plate and incubated overnight at 37 °C. Then, colony PCR was performed on several of the CPEC product colonies and gel electrophoresis was applied to check the size of the pKVS-*vgb*-GFP plasmid. A 0.7 % agarose gel was run for 75 minutes at 82 volts.

fnr Testing in MG1655 Strain

The *E. coli* strains DH5α, BL21 and MG1655 were screened for any deletions in the *fnr* gene. A version of each strain without a GFP plasmid and a version with the FNR-GFP plasmid were tested. The standard protocol for colony PCR was used for each *E. coli* strain to amplify a product of 902 base pairs. Gel electrophoresis was completed to verify if the *fnr* gene was amplified correctly and present in each strain. The amplified DNA from the PCR reaction was ran on a 0.7% agarose gel at 83 volts for 75 minutes.

The MG1655 strain that is *fnr+* was ordered from the Coli Genetic Stock Center and stored in a 15% glycerol stock to test in the future.

Results

Fluorescence Experiments

The reporter protein, GFP, was selected so that the promoter activity of the P_{FNR} could be measured and analyzed quantitatively in aerobic and anaerobic conditions. Therefore, the greater the amount of GFP that was expressed the more effective the promoter was at operating under the given conditions. The first trial of the DH5α, the MG1655 strain trial and BL21 strain trial used an excitation wavelength of 490 nm and an emission wavelength of 510 nm, but the second trial of the DH5α strain used an excitation wavelength of 485 nm and an emission wavelength of 520 nm. An emission scan with a fixed excitation wavelength was completed on the pTrc-GFP plasmid, the PKVS-J23101-GFP plasmid, the BL21 strain and the DH5α strain. The new emission wavelength of 520 nm was selected because at that value the pTrc-GFP plasmid was producing a maximum amount of GFP and the DH5α and BL21 strains showed an insignificant amount of background fluorescence. If the incorrect emission wavelength is used then the negative controls of the fluorescence experiments appear to produce a high amount of background fluorescence due to interference of the plate reader (Attachment 2). This occurred in the first trial of the DH5α strain and the negative control showed high normalized fluorescence values (Figure 1). The adjusted wavelengths decreased the background fluorescence levels in the negative control and the relative values between the positive control, promoter, and negative control were more as expected in the second trial of the DH5α strain. The normalized fluorescence in the positive control is higher and the normalized fluorescence in the negative control is lower when compared to the promoter being tested

(Figure 2). The BL21 strain experiment shows a high normalized fluorescence in the negative control and a higher normalized fluorescence under anaerobic conditions when compared to aerobic conditions of the promoter plasmids (Figure 3). The MG1655 strain experiment also shows a high level of background fluorescence in the negative control, but inconclusive results on higher normalized fluorescence under anaerobic condition (Figure 4).

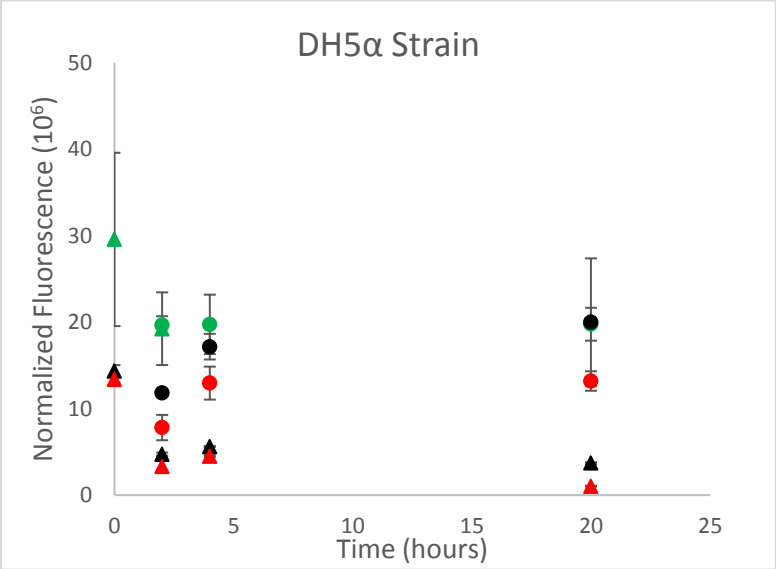
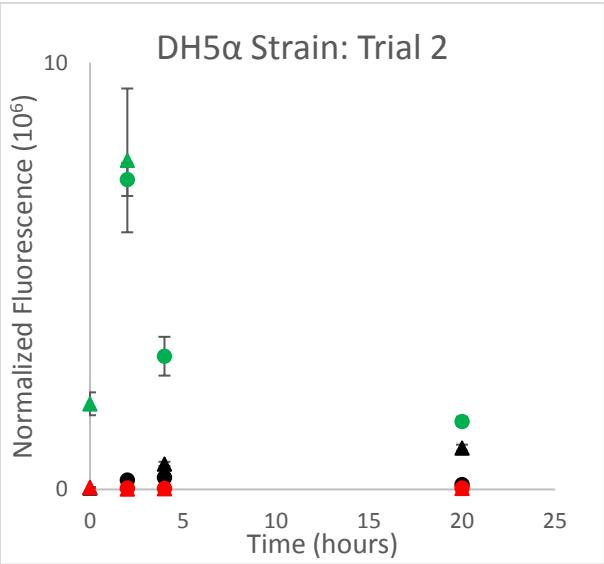


Figure 1. The positive control pTrc-GFP plasmid (green), experimental FNR-GFP plasmid (black) and the negative control that does not contain a plasmid (red) were all tested in the DH5α strain and normalized fluorescence is shown. The triangular markers represent aerobic conditions and the circular markers represent anaerobic conditions. Each error bar represents one standard deviation.

A)



B)

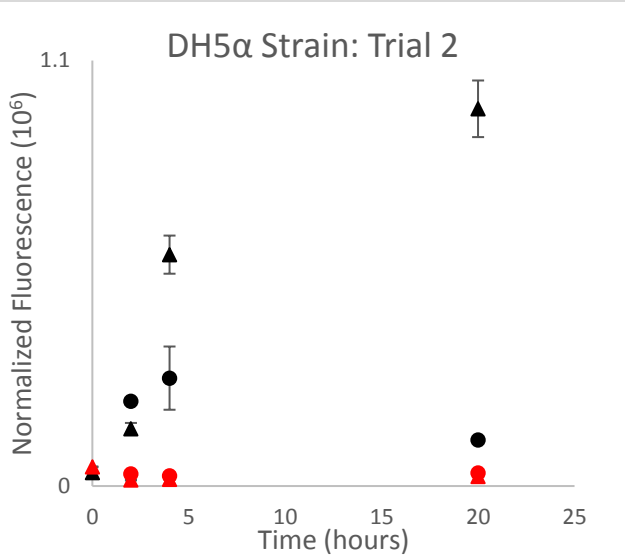


Figure 2. The positive control pTrc-GFP plasmid (green), experimental FNR-GFP plasmid (black) and the negative control that does not contain a plasmid (red) were all tested in the DH5α strain and normalized fluorescence is shown. The triangular markers represent aerobic conditions and the circular markers represent anaerobic conditions. The fluorescence expression during this trial was measured using an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The data points for the aerobic conditions of the positive controls at 4 and 20 hours do not appear on this graph because the plate reader reached saturation under the medium sensitivity setting used. The graph on the right (B) shows just the FNR-GFP plasmid and the negative control on a smaller scale. Each error bar represents one standard deviation.

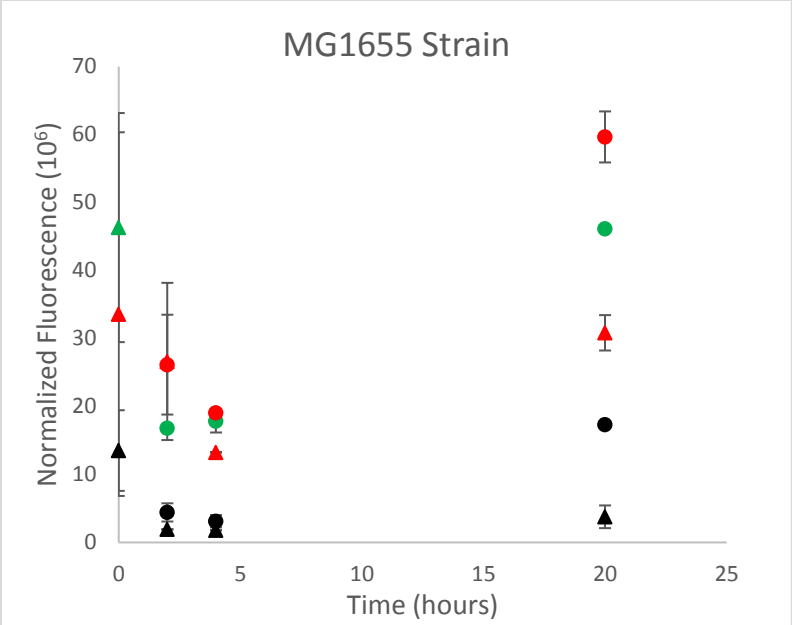


Figure 3. The positive control pTrc-GFP plasmid (green), experimental FNR-GFP plasmid (black) and the negative control that does not contain a plasmid (red) were all tested in the MG1655 strain and normalized fluorescence is shown. The triangular markers represent aerobic conditions and the circular markers represent anaerobic conditions. Each error bar represents one standard deviation.

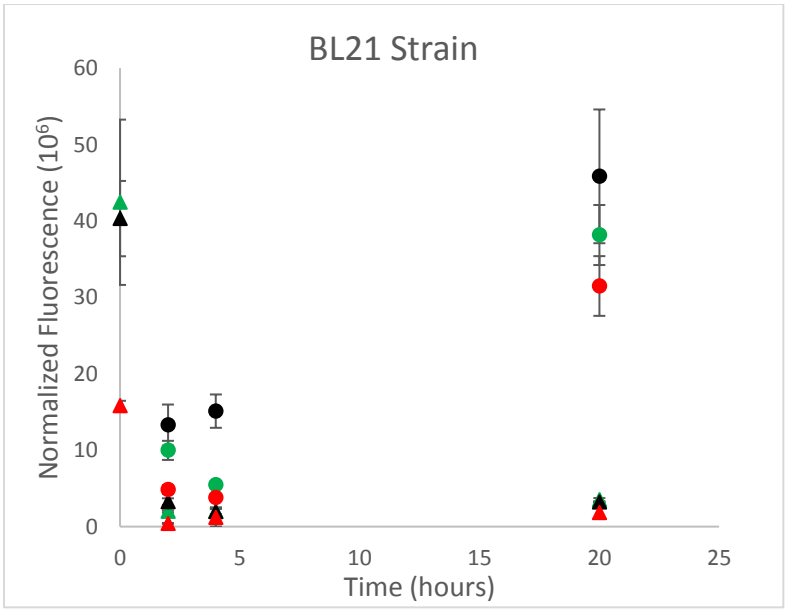


Figure 4. The positive control pTrc-GFP plasmid (green), experimental FNR-GFP plasmid (black) and the negative control that does not contain a plasmid (red) were all tested in the BL21 strain and normalized fluorescence is shown. The triangular markers represent aerobic conditions and the circular markers represent anaerobic conditions. Each error bar represents one standard deviation.

The first trial of the DH5α strain showed that the P_{FNR} is more fluorescent under anaerobic conditions and the positive and negative control have correct relative values by expressing more and less GFP than the P_{FNR}, respectively. Then, in the second trial of the DH5α strain new excitation and emission wavelengths were used, which lowered the background fluorescence levels of the negative control, but cells with the FNR-GFP plasmid did not consistently show a higher normalized fluorescence under anaerobic conditions. Next, the MG1655 trial did not show conclusive results because the experimental FNR-GFP plasmid showed lower normalized fluorescence values than both the positive and negative control. These results lead to the verification of the *fnr* gene in all three strains. Finally, the BL21 strain produced a large amount of GFP with the P_{FNR} under anaerobic conditions, but the positive control showed a lower fluorescence than expected.

pKVS-J23101-GFP and pKVS-vgb-GFP Plasmid Construction

The pKVS-J23101-GFP and pKVS-vgb-GFP plasmids have been constructed using CPEC, but have not been verified as the correct size. Only a miniprepped plasmid of the pKVS-J23101-GFP plasmid shows a strong band through gel electrophoresis, but the PCR reaction did not show the correct segment of DNA being amplified. Also, the double digest on the pKVS-J23101-GFP plasmid did not yield the correct results. Similar results were found for the pKVS-vgb-GFP plasmid and the plasmid has not be verified through gel electrophoresis.

fnr Testing in MG1655 Strain

The MG1655 strain can have large deletions around the *fnr* gene and contain either the *fnr+* or *fnr-* genotype. The stocks of the MG1655 strain with the *fnr-* genotype would not be able to grow under anaerobic conditions. [7] The amplified PCR product of 902 base pairs of the *fnr* gene did not show in the gel electrophoresis test for the MG1655 strain without GFP or the MG1655 strain with the FNR-GFP plasmid. All other strains showed the correct product in the version of the strain without GFP and in the version of the strain with the FNR-GFP plasmid (Attachment 3).

Conclusions and Future Work

The P_{FNR} expresses a larger amount of GFP under anaerobic conditions in the BL21 strain of *E. coli*, while the DH5α and MG1655 strains give inconclusive results. The BL21 strain showed higher normalized fluorescence values when the cells with the FNR-GFP plasmid grew under anaerobic conditions. The first trial of the DH5α strain also showed that the FNR-GFP plasmid under anaerobic conditions produced higher normalized fluorescence levels. However, the second trial of the DH5α strain did not consistently show a higher normalized fluorescence under anaerobic conditions. Also, the second trial of the DH5α strain showed lower levels of background fluorescence due to the change in excitation and emission wavelengths of the plate reader. The DH5α strain should be tested again with the new emission and excitation wavelengths since both trials gave different results for the FNR-GFP plasmid. Then, the MG1655 strain also gave inconclusive results because the stock used had the *fnr-* genotype and does not grow well under anaerobic conditions.

The Gemini plate reader's excitation and emission wavelengths originally selected seemed to have caused interference with the fluorescence measurement and abnormally large background fluorescence levels in the negative controls. The pKVS-vgb-GFP and pKVS-J23101-GFP plasmids are not the correct

size or have not been constructed correctly and will need further testing. Finally, the MG1655 strain was concluded to be the *fnr-* version and the *fnr+* was purchased to be used in future experiments.

The fluorescence experiments can be continued using the new excitation and emission wavelengths that prevent interference from the excitation wavelength on the plate reader. Additional experiments can be performed to test the *fnr+* version of the MG1655 strain and to evaluate all of the *E. coli* strains in Wilms media. Other promoters such as the *vgb*, *gadB* and *pfl* can also be tested in a variety of strains and media. In the future, the pKVS-vgb-GFP plasmid could be constructed using two CPEC reactions to join first the *vgb* and GFP PCR product and then add on the pKVS backbone. Also, additional colonies of the pKVS-J23101-GFP plasmid should be screened for the correct plasmids and diagnostic test such as enzyme digestion can be repeated to verify the results.

Attachments

[1] Reizman – strains and plasmids.xlsx – Tables of all strains, plasmids and primers used

[2] CM Emissions Scans.docx – Emission scans at excitation wavelength of 485 nm on Gemini plate reader

[3] CM fnr Gene Test Report.docx – Report sent to MIT labs on verifying *fnr* mutation in MG1655 strain

[4] CM Protocol for Fluorescence Experiments.docx – Updated protocol for fluorescence experiments

[5] CM Raw Data of Fluorescence.xlsx – Data from each fluorescence experiment, each Excel sheet is a new experiment

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