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WASHINGTON UNIVERSITY IN ST. LOUIS

School of Engineering and Applied Science

Department of Energy Environmental and Chemical Engineering

Thesis Examination Committee:
Fuzhong Zhang, Chair
Tae Seok Moon
Yinjie Tang

Engineering Biosensors for Short-chain Alcohols

by

Yu Xia

A thesis presented to the School of Engineering
of Washington University in St. Louis in partial fulfillment of the
requirements for the degree of
Master of Science

August 2014

Saint Louis, Missouri

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Yu Xia

Washington University in St. Louis
August 2014

Dedicated to my parents.

ABSTRACT OF THE THESIS

Engineering Biosensors for Short-chain Alcohols

by

Yu Xia

Master of Science in Chemical Engineering

Washington University in St. Louis, 2014

Research Advisor: Professor Fuzhong Zhang

Biofuel is a promising substitute for fossil fuel and the research of biofuel production has been extensively conducted during the recent years. Great efforts have been made to create many types of the fuel production hosts. However, effective approaches for high specificity and high throughput screening of the fuel production strains are still lacking. The cellular stress response is one universal defense mechanism when a microbial cell is exposed to an unfavorable substance or environment. It triggers a series of downstream responses when a cell senses certain chemicals. Transcriptional factors are widely used in living organisms to regulate gene expression. They can recognize and bind to specific DNA sequences on the promoter region to repress or activate the DNA transcription. One of the applications in actual practice is to create biosensors using these mechanisms. The biosensors can be designed to detect specific fuel molecules and become useful screening tools in metabolic engineering research and industrial production area. In this study, I demonstrated a novel method for biosensor design based on cellular stress response and the recent developments in genetic circuits.

Chapter 1

Introduction

Biofuels come from renewable sources such as biomass, photosynthetic products, or fermentation products. These resources are nearly carbon neutral, and the use of biofuel will significantly reduce the new carbon emission that petroleum fuel does. Biofuels are in general free of sulfur after combustion, alleviating the environment burden, particularly when used in automobiles [1]. Alcohols with short alkyl chains (C2-C8) are attractive biofuel candidates. Ethanol, which comes from microbial fermentation, can be added to gasoline to a limited extended because of its lower energy density (Figure 1.1). Special engine design is needed to use high ethanol fuels such as E-85 or E-100. Butanol has an energy density closer to gasoline (Figure 1.1). and can be used in current gasoline engines with a higher blend [2]. Compared to ethanol, butanol is more hydrophobic and can be transported through existing pipeline systems [3]. The increasing environmental concerns and the fear of the energy crisis have stimulated pressing needs for renewable energy sources [4]. As one of the alternative fuel solutions, intense research towards the production of short-chain alcohols [5, 6, 7] is underway globally. Microbial production of alcohols avoids the use of high temperature and high pressure which are often needed during chemical processes, and minimizes the waste generation. Furthermore, a microbial-based strategy offers an opportunity to use low-cost, non-food based material (such as lingocellulosic biomass, or CO_2) as feedstock, solving one of the global food problems (ethanol is traditional fermented from corn) [4, 6]. Currently, extensive efforts have been put into the creation of diverse libraries of alcohol-producing strains for high titer production [8, 9, 10, 11]. However, the biofuel research field remains severely impeded by the ability to screen for the best alcohol-producing cell from a large library of native or engineered strains in a high throughput manner. Effective screening for improved microbes has become a very important task in the biofuel research field.

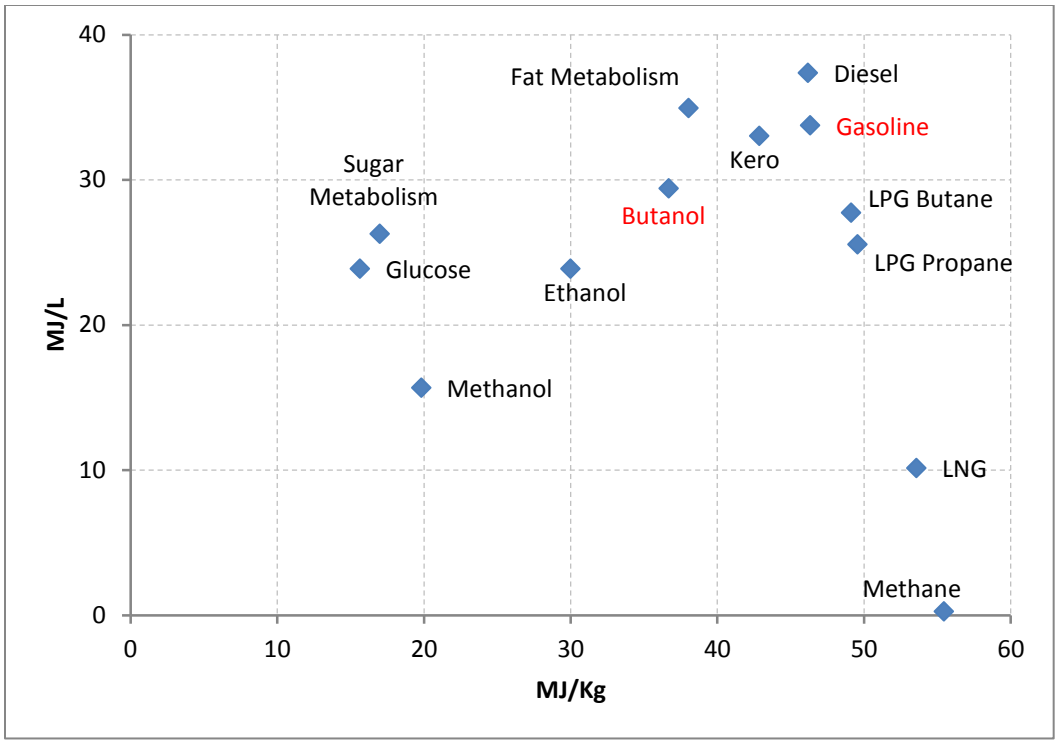


Figure 1.1 Energy density of selected substances.

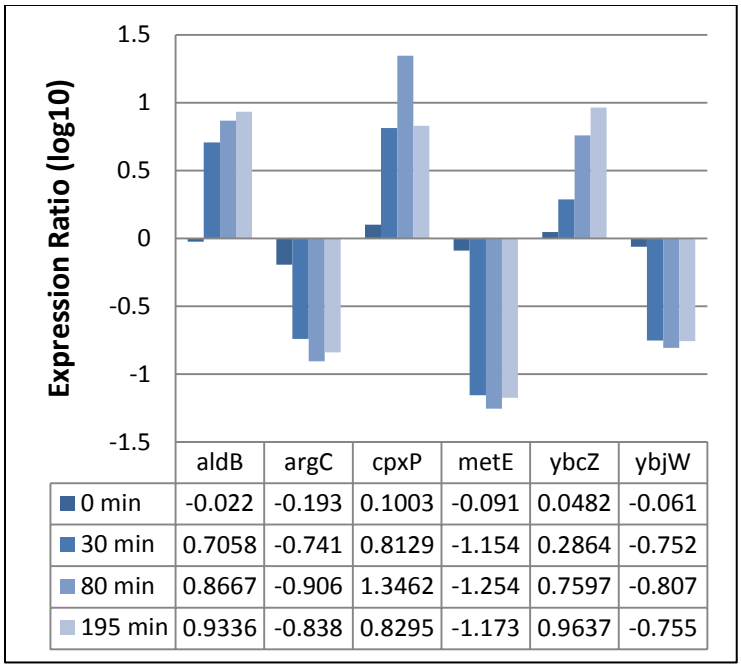
1.1 Objectives

A powerful tool for screening microbial strains is cellular biosensor. Cellular biosensors are cellular components made by engineered microbes that produce measureable signals upon the detection (contact/reaction with) of target molecule [12]. When coupled with proper selection methods such as fluorescent labels and microarrays, cellular biosensors can enable high throughput screening of native or engineered strains. With instrument such as flow cytometers, up to 10^9 cells can be analyzed by fluorescence-activated cell sorting (FACS) per experiment [13]. To date, cellular biosensors for short-chain alcohols are very limited, and the existing biosensors are suffering from low specificity and small dynamic range (alcohol-activated signal vs. inactivated signal). Our work aims to both develop novel design principles and create a series of cellular biosensors with high specificities and large dynamic ranges for short-chain alcohols. These biosensors can be used for high throughput screening of alcohol-producing microbial strains.

1.2 Research Design

The mechanism of our cellular biosensors is based on cellular stress responses and the functions of transcriptional factors. The methodology is based on the recently developed synthetic biology studies.

The cellular stress response is one universal defense mechanism when a microbial cell is exposed to an unfavorable substance or environment. Many different cellular stress responses have been discovered and studied during the past decades. When alcohol is presented or intracellularly produced, native stress responses are turned on due to the toxic nature of the molecule [14]. One universal stress response is the promoter activity change, which is usually a result of the activation of repair or deformation of the damaged essential macromolecules such as proteins and DNA [15]. The promoter activity change can be captured by genome-wide transcriptional analysis, such as RNA microarrays [16]. A recent study has shown that accumulation of metabolite to an excessive level can cause the activity of a group of promoters to change (either up-regulated or down-regulated), as reflected by their controlled transcripts (Figure 1.2). Based on our analysis, 39 *Escherichia coli* (*E. coli*) promoters were only up-regulated (changes > 2 fold and $P < 0.01$) and 30 promoters were only down-regulated when treated with butanol. If any of these promoters are cloned to control the expression of a reporter gene, the reporter's signal could be used as an indicator of the metabolite.



	aldB	argC	cpxP	metE	ybcZ	ybjW
0 min	-0.022	-0.193	0.1003	-0.091	0.0482	-0.061
30 min	0.7058	-0.741	0.8129	-1.154	0.2864	-0.752
80 min	0.8667	-0.906	1.3462	-1.254	0.7597	-0.807
195 min	0.9336	-0.838	0.8295	-1.173	0.9637	-0.755

Figure 1.2 Perturbed activities of selected promoters in the presence of butanol (vary by time). Butanol sensing promoters' activity changes show consistency over a considerable time span.

However, there are several problems with sensors designed using a single promoter: i) many cellular stress responses are based on cell's responses to macromolecular damage in the cell rather than on specific stressors. The response from a single promoter may not be specific to the metabolite because other chemicals may introduce the same damage to the cell and cause the same response. The cell response to the damage regardless of what type of stressor is causing the damage [15]; ii) naive promoters are not tight, and their base level of expression gives the sensors large and unstable background noise even when the metabolites are not present. This is a common problem when dealing with the native promoters. The function of a native promoter is usually embedded with the existing metabolic pathways. An extra copy of the native promoter that controls our interested gene is still complying with its original regulating pathways; iii) in nature, the strength of the cellular regulation is subtle, thus it is very difficult to trigger a significant activity change of the promoter and the dynamic range of the single-promoter-sensor is usually limited. Before solving these problems, it is difficult to apply single-promoter-sensors for metabolite detection.

Fortunately, for the first problem, recent genome-wide transcriptional analysis has demonstrated that ethanol and butanol can change activities of many *E. coli* promoters differently [17] (Figure 1.3).

For example, there are at least 69 butanol-–sensitive promoters had no significant activity change when treated with ethanol. The collective pattern of promoter activity changes is a signature specific to the metabolite that causes stress responses [18]. This result could potentially provide us a strategy of sensor design. If outputs from several promoters are integrated to control the expression of a reporter gene, biosensors specific to the metabolite can be created.

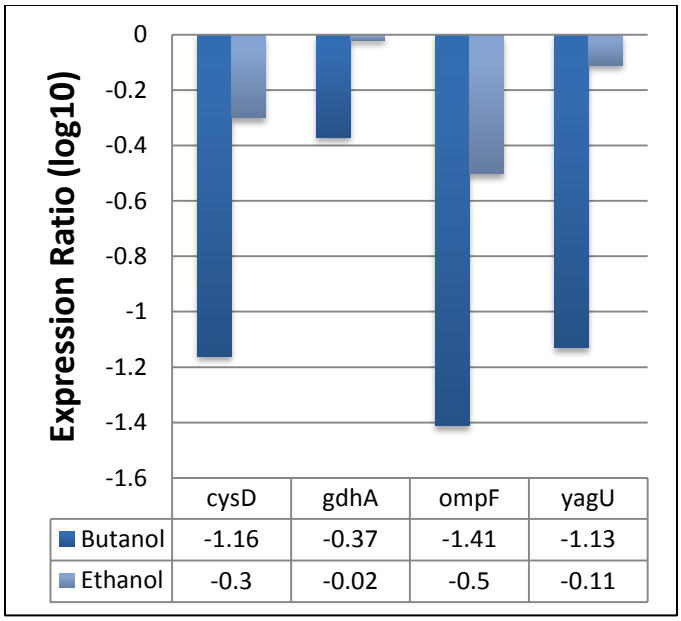


Figure 1.3 Perturbed activities of selected promoters in the presence of butanol and ethanol. Promoters show higher activity change when butanol is in presence than ethanol. Promoter activities are down regulated.

Recent developments in synthetic logic circuits have provided elaborate information-processing capabilities and precise control of gene expression over large output ranges [19, 20]. Hence, we aim to engineer logic circuits that integrate signals from multiple promoters to control a reporter gene, whose output signal is governed by the concentration of butanol, can be created.

Several genetic circuits [21] have been previously created, enabling computation of complex logic, including AND, OR, NOT, NOR, and XOR functions in living cells. Of particular interesting to this project is the AND logic gate that allows integration of multiple inputs by inserting multiple operate sites into one promoter to block transcription. An AND logic gate means only when all the inputs are satisfied, the expected output would be presented (Table 1.1).

Table 1.1 Truth Table of an AND Gate Logic System

INPUT		OUTPUT
A	**B**	**C**
0	**0**	**0**
1	**0**	**0**
0	**1**	**0**
1	**1**	**1**

In our study, transcription factors are used in the design of the AND gate. A transcription factor is a protein that binds to specific DNA sequences. Transcription factors can either enhance or repress gene expression by promoting or blocking the RNA polymerase from binding to the DNA (Figure 1.4).[22] The advantage of using transcriptional factors is that they are abundant and can be easily evolved or re-engineered based on specific needs.

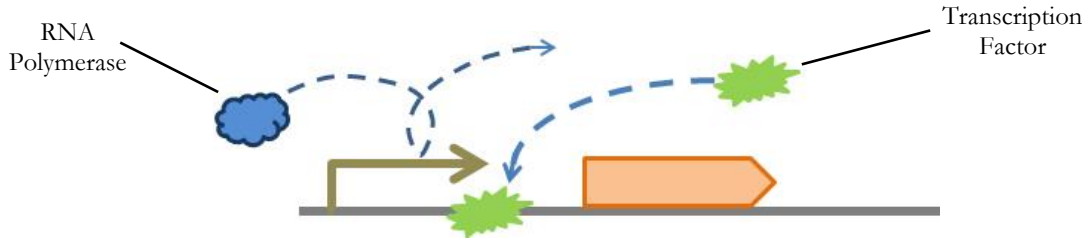


Figure 1.4 Example of the function of a transcription factor. In this example, a transcription factor (repressor) binds to a designated DNA binding domain on the promoter region and blocks the RNA polymerase from binding to the DNA. The transcription is thus repressed.

Promoter activity regulated by multiple repressors is common in nature and well-studied on synthetic promoters. Placing repressors that bind to DNA sequences between the RNA polymerase binding site and the start codon is expected to interfere with transcription [23]. The interference will be fully removed only when all the repressors are deactivated from the cell. I choose engineered zinc finger proteins (ZFPs) and their corresponding DNA sequences as repressors and operators because zinc fingers are amenable to fine tuning and have been extensively used in regulating synthetic circuits.

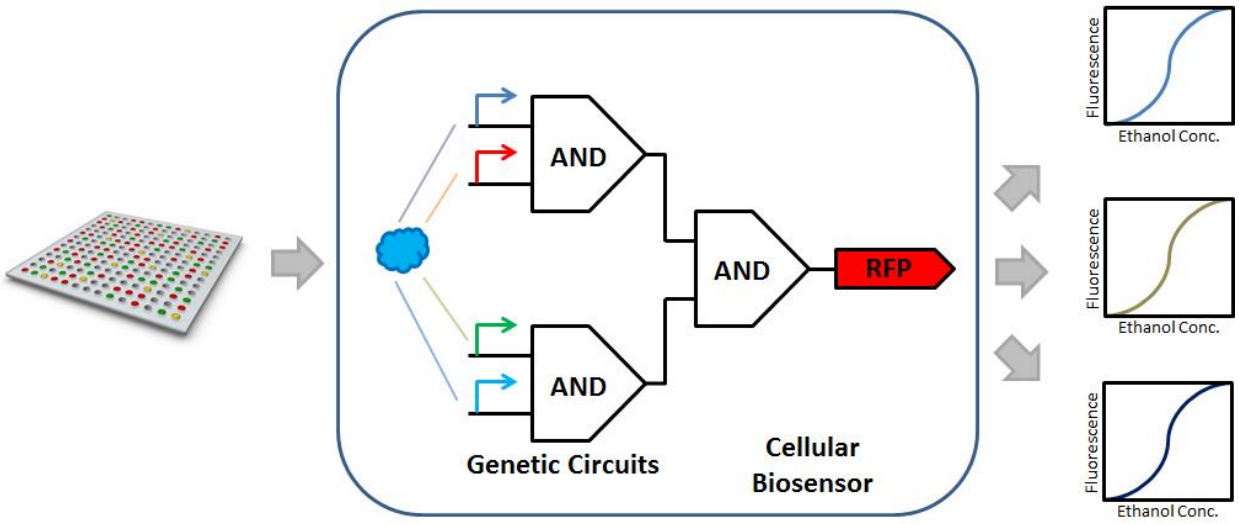
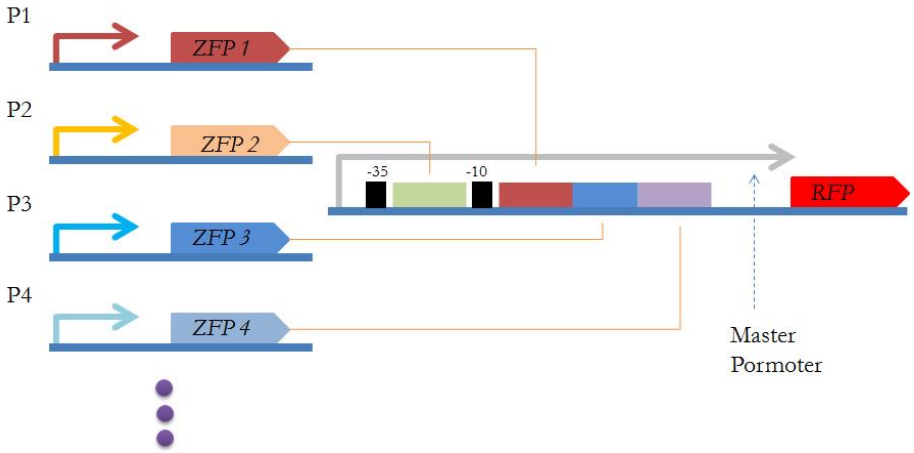


Figure 1.5 Microbial biosensors for the detection of short-chain alcohols. RNA microarray will be performed for cells treated with individual alcohol. Promoters that are specifically regulated by alcohol stress will be identified and integrated using AND logic circuits to control an output signal. Using this design principle, I aim to develop several microbial strains that detect individual short-chain alcohols.

In our study (Figure 1.5), a negative butanol-ZFP-controlled mechanism (ZFP's concentration decreases with butanol concentration) is created by using butanol down-regulated promoters. Negative regulations are useful in metabolite biosynthesis and selection when the regulation is used to control a protein toxin [24]. To do so, several butanol down-regulated promoters are cloned to control the zinc fingers. In this system, zinc fingers under the control of down-regulated promoters are integrated to recognize and bind to the operator sites on a synthetic promoter, which controls the expression of a final reporter gene. In our design (Figure 1.6), the butanol specific, down-regulated promoters (P1, P2, P3 and P4 in Figure 1.6) will be cloned to control a set of *zfp* genes. The ZFPs are orthogonal repressors. The corresponding operator sites of these repressors are then integrated into the synthetic promoter (P_{SynBu}) to control the expression of a reporter, red fluorescent protein (RFP). Such a design principle is expected to perform as an AND logic gate [25]: in the absence of butanol, repressors turn the synthetic promoter off, and RFP is not expressed; when butanol concentration increases, the butanol down-regulated promoters' activity decreases, and the all the ZFPs are specifically and simultaneously down-regulated, leading to de-repression of the synthetic promoter and increased RFP fluorescence.



Alcohol Level	P1…P4 Activity	ZFP Expression Level	Repressor Level	Master Promoter Repress Level	Master Promoter Activity	RFP Expression Level	RFP Intensity
↑	↓	↓	↓	↓	↑	↑	↑

Figure 1.6 Example of a butanol biosensor using AND logic circuit. Increased butanol concentration turns down the expression of several orthogonal repressors (such as Zinc Finger Proteins) through promoters (i.e. P_{ompF}) identified by butanol stress. Decreased repressor levels then turn up RFP expression.

Chapter 2

Materials and Methods

2.1 Materials

T4 DNA ligase and restriction endonucleases were obtained from New England Biolabs. Phusion High-Fidelity PCR Kit, GeneJET Plasmid Miniprep Kit, GeneJET Gel Extraction Kit, GeneJET Genomic DNA Purification Kit, and GeneJET PCR Purification Kit were purchased from Thermo Scientific. All other reagents were from Sigma Aldrich. Mini gene and oligonucleotide primers were synthesized by Integrated DNA Technologies (IDT). Gene sequencing was performed by Protein and Nucleic Acid Chemistry Laboratory (PNACL), Washington University in St. Louis.

2.2 Plasmid Construction

The plasmids used or constructed in this study are listed in Table 2.1. The *SynBu* promoter was prepared synthetically by IDT (Figure 2.1). And the synthetic promoter (*synbu*) was amplified with PCR. The PCR product was purified, digested with *Aat*II and *Eco*RI and ligated into *Aat*II and *Eco*RI digested plasmid pB5a-RFP to construct pBSynBua-RFP. The native promoters *cysD*, *ompF*, *gdhA*, *yagU*, *ybcZ*, *cpxP*, *aldB*, *argC*, *metE*, and *ybjW* were cloned from the genomic DNA of *E. coli* K-12 MG1655 strain by PCR. The PCR products were purified, digested with *Eco*RI and *Bam*HI and ligated into *Eco*RI and *Bam*HI digested plasmids pS5c-RFP and pE5c-RFP to replace the *lacUV5* promoters from both plasmids. DNA sequences of two degradation tags, *asv* and *aav,* were separately added to the C-terminal of the fluorescent protein (*gfp*) genes by PCR or Golden Gate Assembly method. A LAA tag designated *yemgfp* gene was cloned from plasmid pJS167cw [26] by PCR. All the digested and purified regular *gfp*, tagged *gfp*, and *yemgfp* gene fragments were digested by *Eco*RI and *Bam*HI for replacing the *rfp*s on the plasmids that have been constructed in our study. The zinc finger protein genes (*zfp-40*, *zfp-62*, *zfp-68* and *zfp-77*) were obtained from plasmids pWH-

29-40, pWH-29-62, pWH-29-68, and pWH-29-77, respectively by digesting with *Eco*RI and *Bam*HI. The digested and purified four *zfp* gene fragments were subsequently ligated to *Eco*RI and *Bam*HI digested plasmid pS5c-RFP and plasmids with native promoters created above to replace the *rfp* gene.

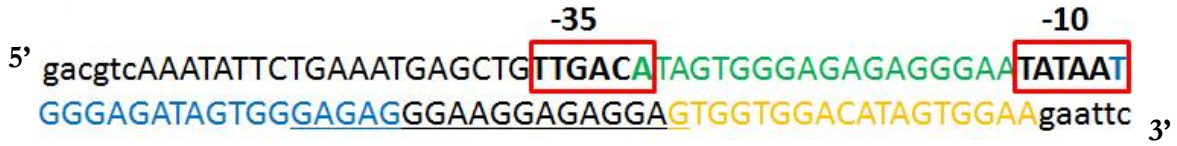


Figure 2.1 Diagram of the synthetic promoter. Green: ZFP-68 binding site; blue: ZFP-62 binding site; underline: ZFP-77 binding site; orange: ZFP-40 binding site.

Table 2.1 Plasmids Used in This Study

Plasmid	Description	Ref.
pBSynBua-RFP	**SynBu promoter, pBBR1 origin, RFP, Amp^R**	**This study**
pE5c-RFP	**lacUV5 promoter, colE1 origin, RFP, Cm^R**	**Zhang et al. (2012)**
pS5c-GFP	**lacUV5 promoter, pSC101 origin, GFP, Cm^R**	**Zhang et al. (2012)**
pS5c-RFP	**lacUV5 promoter, pSC101 origin, RFP, Cm^R**	**Zhang et al. (2012)**
pJS167cw	**lacARA1 promoter, colE1 origin, RFP, Kan^R**	**Hasty et al. (2006)**
pWH-29-ZFP	**pBAD promoter, pSC101 origin, ZFP (40,62,68 or 77), Cm^R**	**Zhang et al. (2012)**
pS(native promoter)c-RFP	**Native promoter (cysD, ompF, gdhA, yagU, ybcZ, cpxP, aldB, argC, metE, or ybjW), pSC101 origin, Cm^R**	**This study**
pE(native promoter)c-RFP	**Native promoter (cysD, ompF, gdhA, yagU, ybcZ, cpxP, aldB, argC, metE, or ybjW), colE1 origin, Cm^R**	**This study**
pS5c-GFP(with tag)	**lacUV5 promoter, pSC101 origin, GFP with AAV or ASV tag, Cm^R**	**This study**
pS5c-RFP(with tag)	**lacUV5 promoter, pSC101 origin, RFP with AAV or ASV tag, Cm^R**	**This study**
pE(native promoter)c-yemGFP-LAA	**Native promoter (cysD, ompF, gdhA, yagU, ybcZ, cpxP, aldB, argC, metE, or ybjW), colE1 origin, yemGFP with LAA tag, Cm^R**	**This study**
pS(native promoter)c-ZFP	**Native promoter (cysD, ompF, gdhA, yagU, ybcZ, cpxP, aldB, argC, metE, or ybjW), pSC101 origin, ZFP (40,62,68 or 77), Cm^R**	**This study**

2.3 *E.coli* Strain

The strains used in this study are listed in Table 2.2. *E.coli* Strain K-12 MG1655 was used to obtain the native butanol sensitive promoters from its genomic DNA. *E.coli* DH10B competent cells were used for all the plasmid constructions. All the sequenced plasmids were retransformed into *E.coli* DH1 competent cells for latter growth measurements.

The X1 series strains (Table 2.2) are DH1 cells with single plasmid that has one *rfp* gene controlled by a native butanol sensitive promoter. The X1T series strains are DH1 cells containing single plasmid that has a tagged fluorescent protein gene controlled by a native butanol sensitive promoter. The X1 and X1T series were used for characterizing the native butanol sensitive promoters. X2 series strains are DH1 cells that transformed with two plasmids. One of the plasmids in an X2 strain has a *zfp* gene controlled by a native butanol sensitive promoter. The second plasmid in an X2 strain is the pBSynBua-RFP plasmid (Figure 2.2). The X2 series was used for testing the negative response sensor design with native promoters. The C1 strain is a DH1 cell that transformed with pS5c-RFP plasmid. The C2 series strains are transformed with a pBSynBua-RFP plasmid and a plasmid that contains one of the *zfp* genes controlled by the Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible promoter *LacUV5*. The C1 and C2 strains were used for control purpose.

Table 2.2 Important Strains Used in This Study

Strains	Genotype	Ref.
MG 1655	**F- lambda- ilvG- rfb-50 rph-1**	**Liu and Reeves 1994**
X1	**pS(native promoter)c-RFP**	**This study**
X1T	**pS5c-GFP(with tag), pS5c-RFP(with tag), or pE(native promoter)c-yemGFP-LAA**	**This study**
X2	**pBSynBua-RFP with pS(native promoter)c-ZFP**	**This study**
C1	**pS5c-RFP or pS5c-GFP**	**This study**
C2	**pBSynBua-RFP with pS5c-ZFP**	**This study**

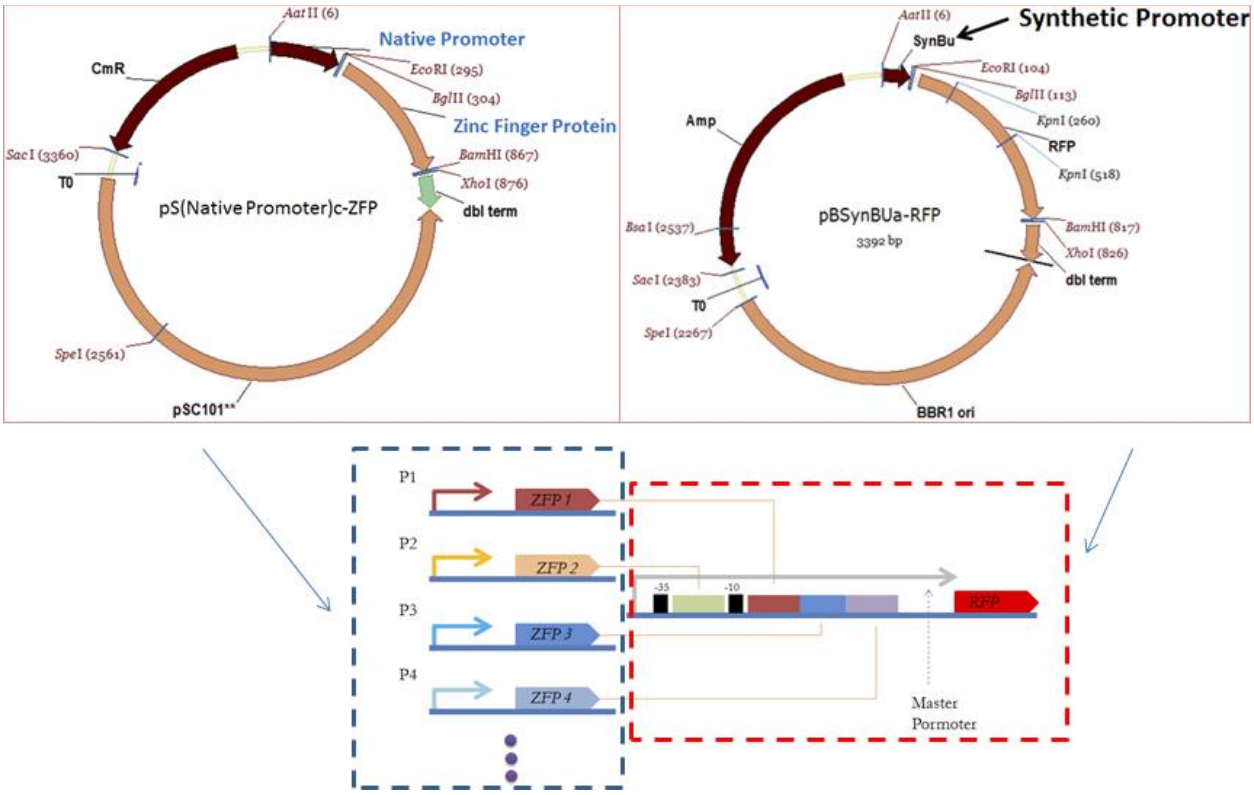


Figure 2.2 Design diagram of X2 strain. In actual construction, the two parts of the sensor design in Figure 1.6 are two plasmids. One plasmid plays the role of the LHS on the diagram and the second plasmid plays the role of the RHS on the diagram.

2.4 Cell Growth

Both Lysogeny broth (LB) and M9 medium were used for cell growth, supplemented with ampicillin (100 mg/ml), chloramphenicol (20 mg/ml) or kanamycin (40 mg/ml) as appropriate. Single colonies of each strain were inoculated into 5ml of LB with appropriate antibiotics and cultured overnight at 37 °C. The seed cultures were diluted to OD_{600} of 0.1 with 10 ml fresh LB medium with appropriate antibiotics and cultivated at 37 °C in a rotary shaker (240 rpm). The cultures were then induced with appropriate concentration of IPTG or Butanol at OD_{600} of 0.6 for time-course measurements as described below. If M9 medium was used, 50 ~100 μL seed cultures from LB medium were then used to inoculate 5 ml M9 medium with appropriate antibiotics and cultured overnight at 37 °C. The overnight M9 cultures were diluted to OD_{600} of 0.1 with 10 ml fresh M9 medium with appropriate antibiotics and cultivated at 37 °C in a rotary shaker (240 rpm). The cultures were then induced with appropriate concentration of IPTG or Butanol at OD_{600} of 0.6 for time-course measurements as described below.(Figure 2.3)

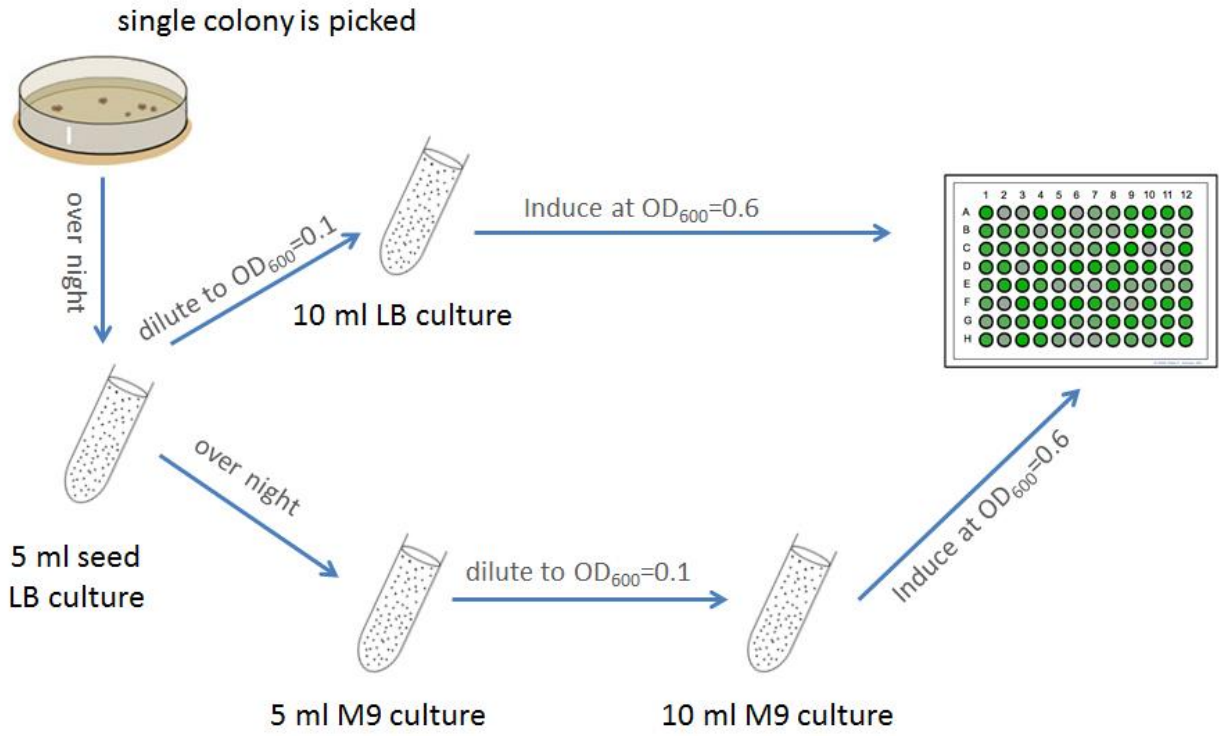


Figure 2.3 Example of Cell Growth Procedure

2.5 Native Promoter Characterization

The X1 and X1T cell lines were induced with different concentrations of Butanol (Figure 2.4) at OD_{600} of 0.6. For time-course measurements of the promoters' responsiveness, samples from various time points were collected and measured with plate reader (Infinite 200 PRO, TECAN) on a 96-well black plate (Falcon). For RFPs with and without the degradation tags, the excitation wavelength was 535 nm and the emission wavelength was 620 nm. For GFPs with and without the degradation tags, and the yemGFP, the excitation and emission wavelengths were 483 nm and 530 nm, respectively. Same wavelengths were used for all the following measurements in our study as shown below. Cultures were grown and measured for 24 hours after being induced with butanol.

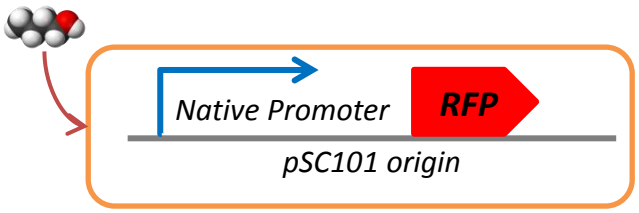


Figure 2.4 Example of native promoter characterization with X1 strain

2.6 Sensor Responsiveness Measurements

The C1 and C2 strains (Figure 2.5) were induced with different concentrations of IPTG at OD_{600} of 0.6. The X2 strains were induced with different concentrations of butanol at OD_{600} of 0.6. The induced cultures were then transferred onto the 96-well black plate for kinetic measurements. The samples were shaken inside the plate reader at 37 °C and measured every 15 minutes until all the cultures reached stationary phase. In our study, only the promoters showed proper responses during characterization were chosen for the X2 strains.

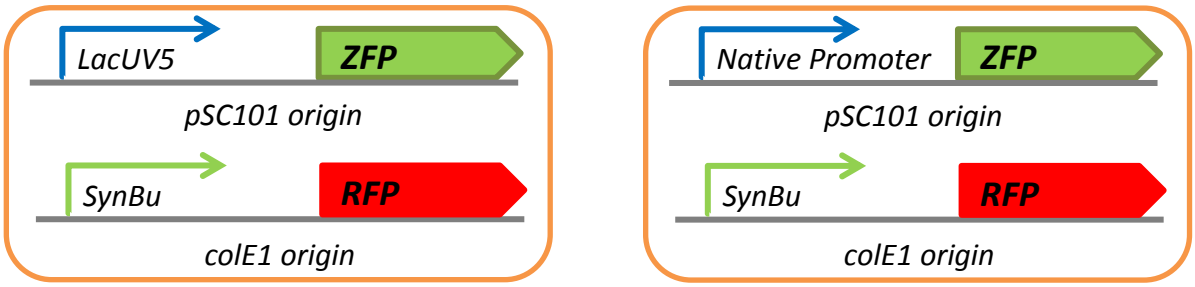


Figure 2.5 Example of sensor responsiveness measurement experiments. C2 strain (left) is induced with different concentration of IPTG and X2 strain (right) is induced with different concentration of butanol.

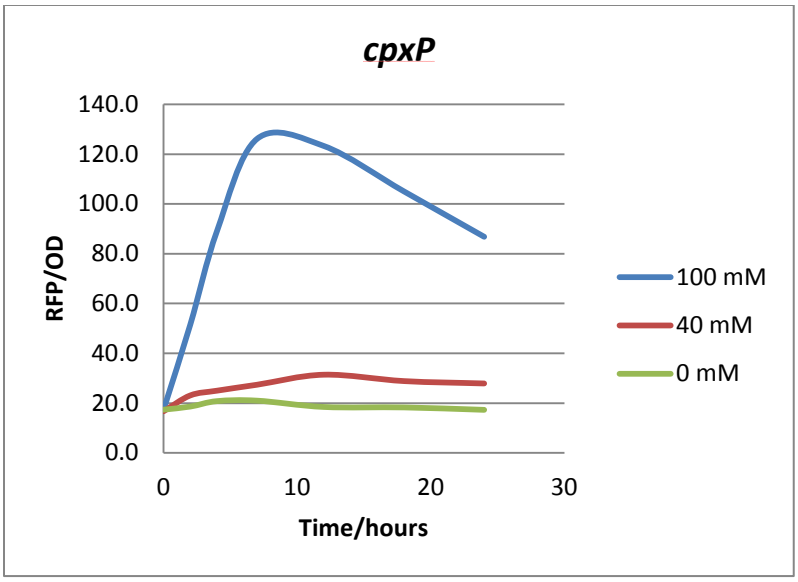
Chapter 3

Results and Discussion

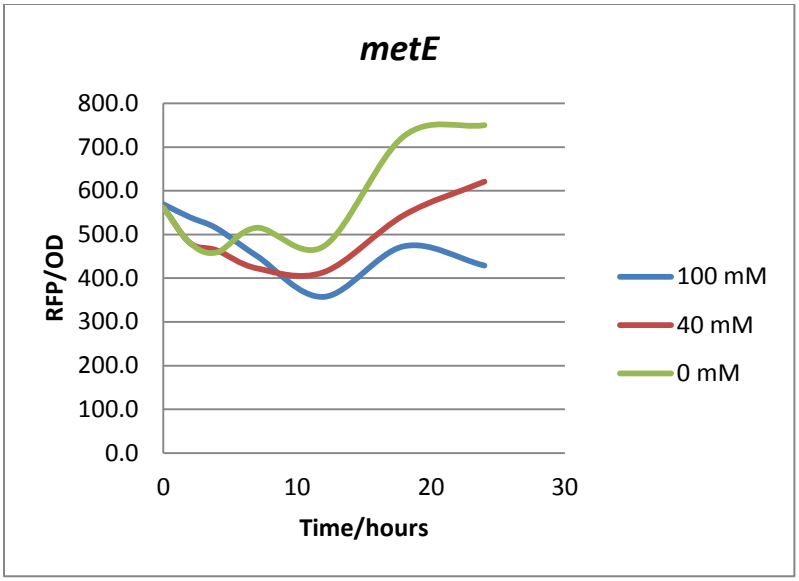
3.1 Results and Discussion

3.1.1 Native Promoter Characterization

To test whether the promoters we chose based on the RNA microarray studies could properly respond to butanol on the protein level when exogenous butanol was added to the growth medium. The *E. coli* X1 strain was grown to exponential growth phase. Different amount of butanol was then added to the medium. RFP was used as the reporter to show the promoters' activity changes. The data from RNA microarray study has provided us with the ratio of change when the promoters were treated with butanol, but the data didn't provide us the strength of each promoter. Delicate change of the promoter's activity could be adequate for cellular regulation, but would not be sufficient for designing a sensor with high specificity and high dynamic range. Among the 10 promoters we chose, signals from promoters *cysD, gdhA, aldB*, and *ybjW* were too weak to be analyzed. Promoters of *ompF, argC, ybcZ*, and *yagU* showed strong signals, but the expected signal change were not observed. A possible explanation is that the transcriptional level change of the mRNA could be transient while the protein expression level is the integral of mRNA level over a long period of time. A transient boost-up on the mRNA level could not cause a significant increase on the accumulated protein number. In addition, after the translation is completed, proteins also need a long time to fold, which make transient signal even harder to be detected. As shown in Figure 3.1, promoter *cpxP* and promoter *metE* showed significant activity changes when butanol was added. Promoter *cpxP*'s activity increased 5 folds in medium containing 100 mM of butanol and increased 2 folds when 40 mM of butanol was contained in the medium (Figure 3.1a). Promoter *metE* decreased by 1.9-fold in medium contained 100 mM of butanol and by 1.6 fold in 40 mM of butanol (Figure 3.1b).



a



b

Figure 3.1 Perturbed gene expression in the presence of different amount of butanol. (a) X1 strain contains pScpxPc-RFP plasmid treated with 0 mM, 40 mM and 100 mM butanol; (b) X1 strain contains pSmetEc-RFP plasmid treated with 0 mM, 40 mM and 100 mM butanol.

Though *cpxP* is an up-regulated promoter, and the strength is comparatively not strong, further study made us believe the data is reliable. The *cpxP* is one of the genes in *E. coli* that respond to cell envelop stress. [27] Butanol causes increasing of membrane fluidity and disrupting of protein-lipid

interactions. The transcription of *cpxP* will be induced upon biofilm formation [28]. A parallel experiment with colE1 replication origin also suggested that promoter *cpxP*'s activity increased upon the detection of butanol (Figure 3.2). We have also discovered that 40 mM of butanol is the upper limit in our case that butanol is not causing any significant disturbance in cell growth.

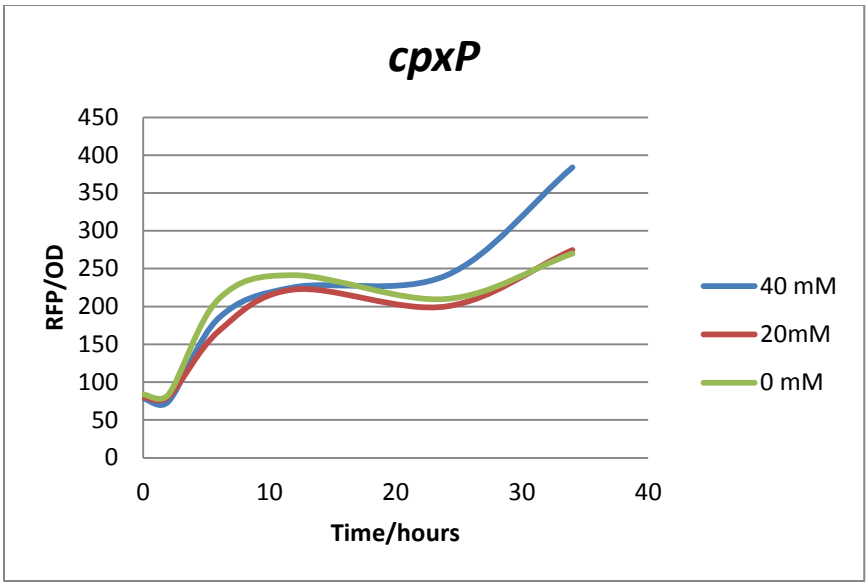


Figure 3.2 X1 strain contains pEcpxPc-RFP plasmid treated with 0 mM, 20 mM and 40 mM butanol.

The ZFP-promoter interaction has a very small dissociation constant ($Kd_{ZFP\text{-}Promoter} \approx 1x10^{-8}M$) [29], theoretically very small amount of ZFP are needed to fully repress the synthetic promoter. Thus a low copy plasmid is preferred for carrying *zfp* genes to achieve a quick response, and a high copy plasmid should be used for carrying the synthetic promoter for better dynamic range.

3.1.2 Sensor Responsiveness Measurements

3.1.2.1 IPTG inducible promoters

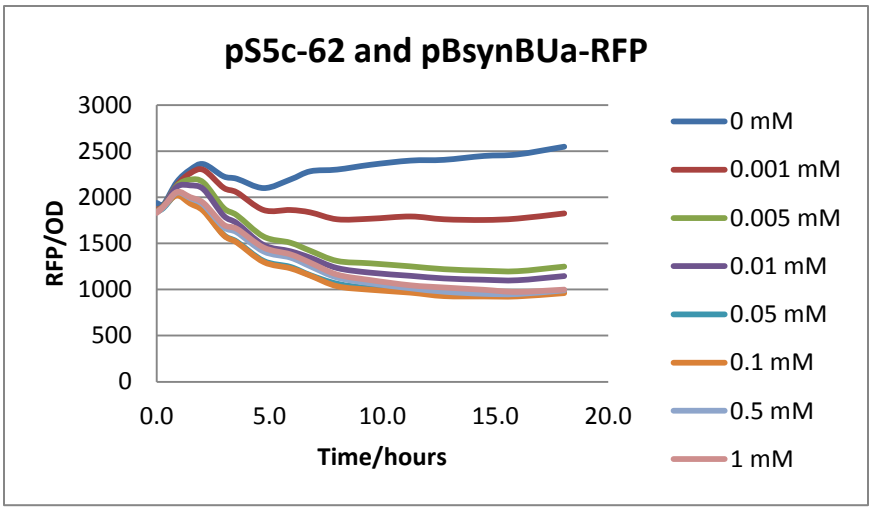
We hypothesized that with a negatively regulated promoter, the increased butanol concentration will down regulate the butanol sensitive promoter's activity, and lower the repressor concentration, so the synthetic promoter's activity will be de-repressed, and the RFP level will be increased. To test this hypothesis, we designed C2 strain. The *LacUV5* promoter is an IPTG inducible promoter that can simulate the activity change of the butanol sensitive promoters. Thus, different repressor level can be achieved intracellularly. The more IPTG is added, the higher *LacUV5* promoter's activity is.

We created four different pS5c-ZFP plasmids with four different zinc fingers, and each one of them was co-transformed into a DH1 cell with a pBSynBua-RFP plasmid. One problem we had in this experiment was mutation, which occurred constantly during the construction of pBSynBua-RFP. A region consists the -10 site, a very critical site for RNA polymerase recognition, was always deleted during the cell growth. Further investigation revealed that two repeated sequences on the two very similar regulator sites on both sides of the -10 site could lead to the deletion of the repeated sequence including the -10 site as the result of a false DNA self-repair during the DNA replication. After removing one of the similar regulator sites, mutation no longer happened (Figure 3.3).

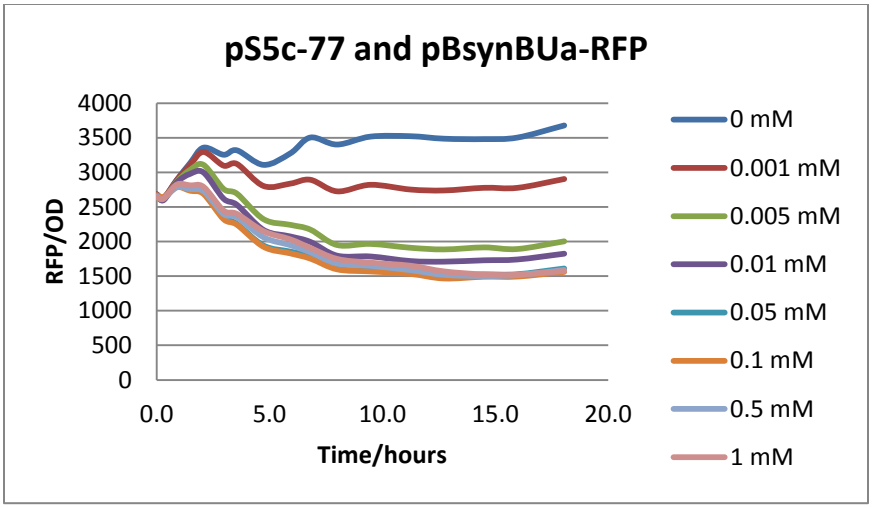
5'……TTTTTTgacgtcAAATATTCTGAAATGAGCTGTTGACA**TGGGAGATAG TGGGAGAG**TATAAT**GAGAGGGAAGGAGAGGAGTGGTGGACATAGTGGAAg aattcTTTTTT……3'**

Figure 3.3 Revised design of the synthetic promoter. Blue is the binding site of ZFP-62; underline is the binding site for ZFP-77; Orange is the binding site for ZFP-40. The binding site of ZFP-68 was removed to avoid the mutations happen.

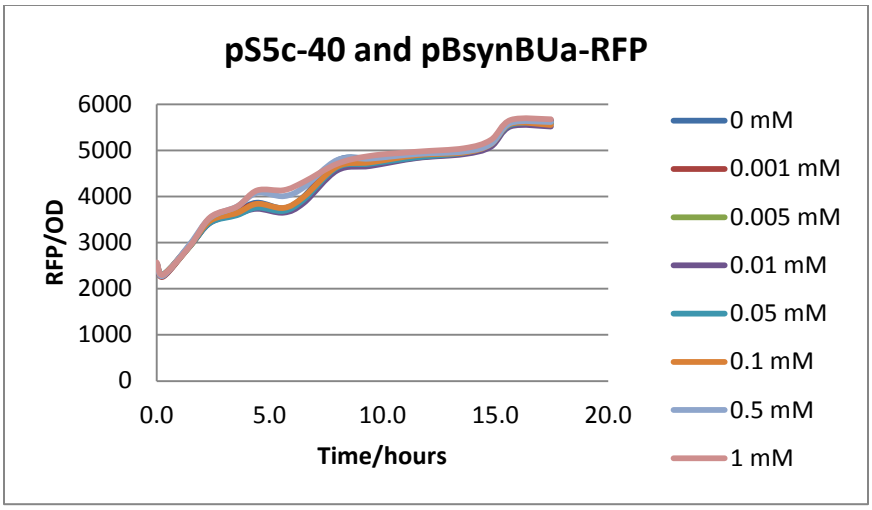
We first tested the C2 strains in LB medium. The strains with ZFP-62 and ZFP-77 showed decreased RFP level when IPTG was added. The result (Figure 3.4) is consistent with our initial hypothesis: when IPTG concentration increases, the *LacUV5* promoter's activity increases, and the ZFP levels inside the cells were up-regulated, leading to the repression of the synthetic promoter and decreased RFP fluorescence. A dose effect could also be observed when different concentration of IPTG is added. The RFP level decreased in gradient with the IPTG concentration gradient. In this experiment, ZFP-40 didn't show any effect, and no repression was ever observed (Figure 3.4c). The leaky expression of the *LacUV5* promoter could also show its effect when the sensor data were compared to that from a ZFP-free system. The initial values of RFP fluorescence (at time zero) in experiments with ZFPs are lower than the experiment with ZFP free cells. This result also suggests very low amount of ZFP in presence could cause perceptible depression of the synthetic promoter's activity. Before being repressed, we could see the RFP fluorescence continued to increase for a short period of time after the cultures were induced with IPTG. This was because the already made, but unfolded RFPs were continue folding though their synthesis had been interrupted. It also suggests that ZFPs take time to synthetize and fold in order to be functioning.


pS5c-62 and pBsynBUa-RFP
RFP/OD
3000
2500
2000
1500
1000
500
0
0.0
5.0
10.0
15.0
20.0
Time/hours
0 mM
0.001 mM
0.005 mM
0.01 mM
0.05 mM
0.1 mM
0.5 mM
1 mM

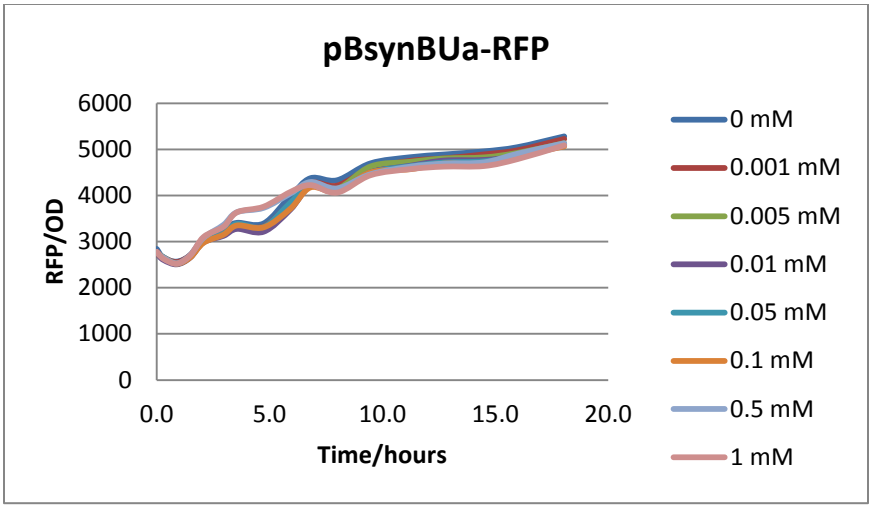
a


pS5c-77 and pBsynBUa-RFP
RFP/OD
4000
3500
3000
2500
2000
1500
1000
500
0
0.0
5.0
10.0
15.0
20.0
Time/hours
0 mM
0.001 mM
0.005 mM
0.01 mM
0.05 mM
0.1 mM
0.5 mM
1 mM

b



c



d

Figure 3.4 Results from the kinetic data of sensor responsiveness measurements in LB medium. Strains are induced with different concentrations of IPTG. (a)(b)(c). C2 strains are transformed with a vector carrying one *LacUV5* controlled ZFP and a vector carrying synthetic promoter controlled reporter gene. (d). A repressor-free cell only transformed with a vector carrying synthetic promoter and its controlled reporter gene.

A data comparison between ZFP-62 and ZFP-77 suggests that the depression level caused by these two repressors were different. The distance between the operator site and the RNA polymerase binding site played an important role here [29]. Based on the synthetic promoter's design, ZFP-62 can block right onto the RNA polymerase binding site on the synthetic promoter, while ZFP-77 binds next to it. So, ZFP-62 showed stronger inhibition capability. Although ZFP-77 binds next to

the RNA polymerase binding site, its steric effects will still strongly affect the RNA polymerase from binding to the promoter. However, ZFP-40 binds far from the RNA polymerase binding site, which makes it incapable to repress the promoter. It suggests that the regulator site's effectiveness is closely related to its distance to the RNA polymerase binding point. If the repressor binding site is distant, unless the protein is big enough or can cause heavy topological structure change to the DNA, the repressor will not be functional.

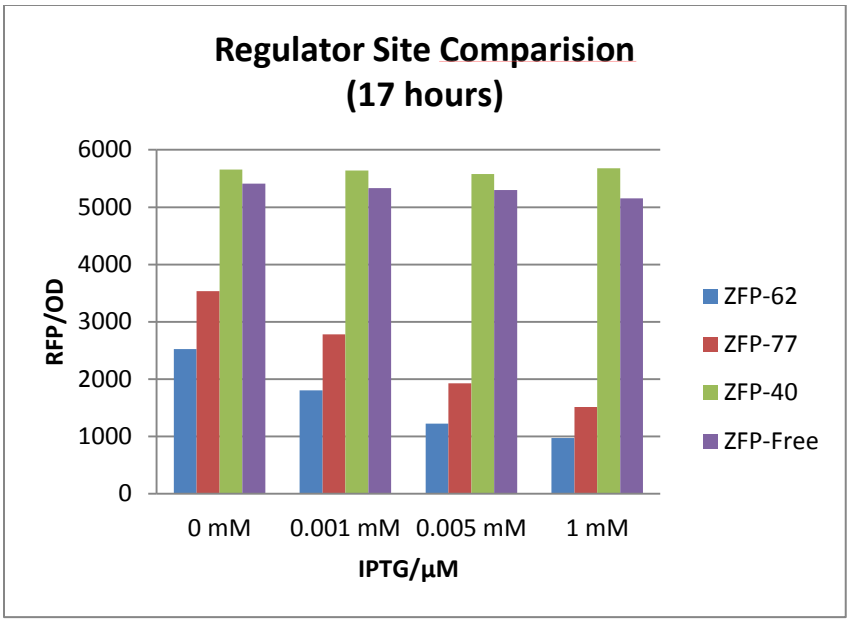
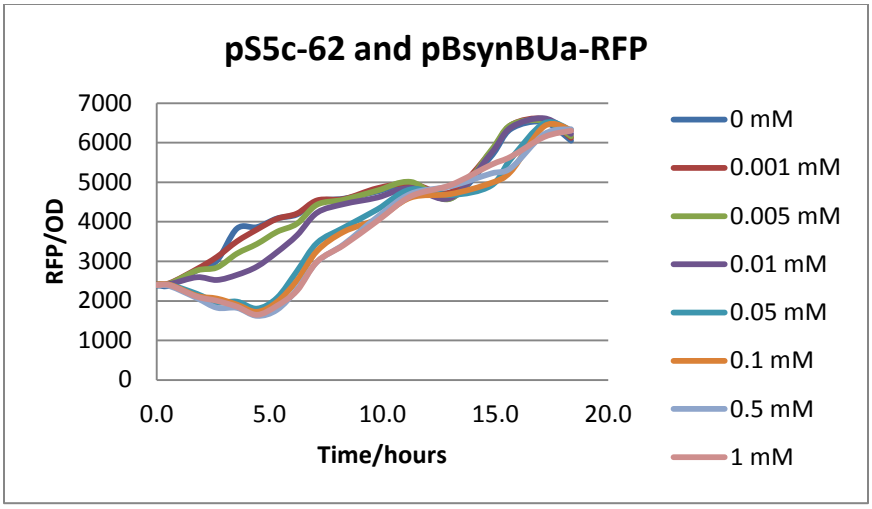


Figure 3.5 Repression results of ZFP at different promoter regulator sites in stationary growth phase.

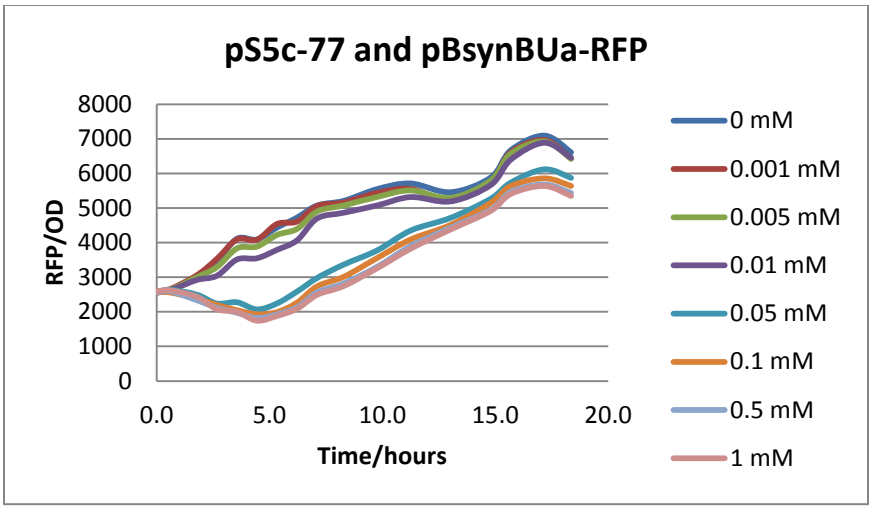
In the following study shown below, only ZFP-62 and ZFP-77 were used.

Same experiments were also performed in M9 medium (Figure3.6). The M9 medium data are polarized. The response did not show an expected progressive change or dose effect as the LB experiments did. The synthetic promoter was heavily repressed in first 5 hours when IPTG concentration was higher than 0.05 mM. However, the inhibition was removed after 5 hours. In M9 medium, which has high glucose, the RFP's synthesis rate is much faster than in the LB medium. Once the cell growth reaches exponential phase (at 5 hours), the ZFPs became incapable to repress the high elevated RFP synthesis rate. I also compared these results to one of our previous pS5c-RFP titration data (Figure 3.7). I found the sudden change of the results between IPTG concentration of 0.01mM and 0.05mM was caused by the *LacUV5* promoter. In M9 medium, the *LacUV5*

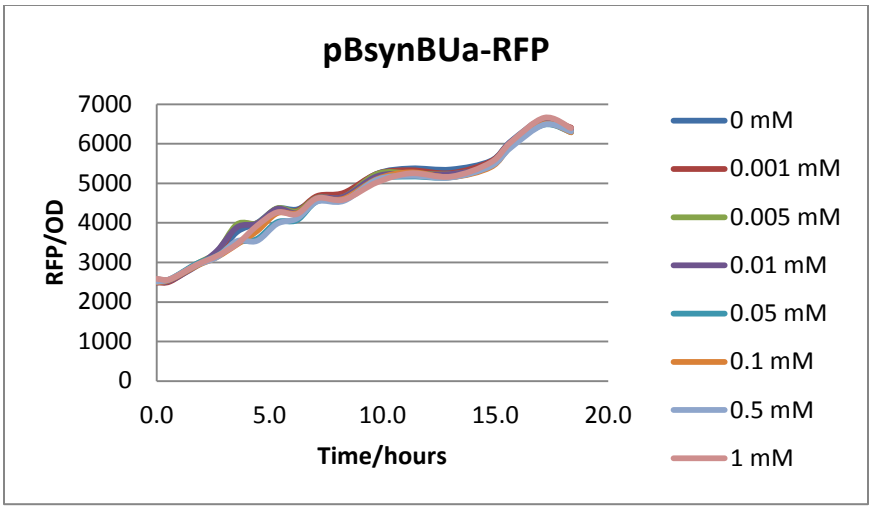
promoter's activity leaped between the IPTG concentration of 0.01 mM and 0.05 mM, and this trend was consistent with the sensor data.



a

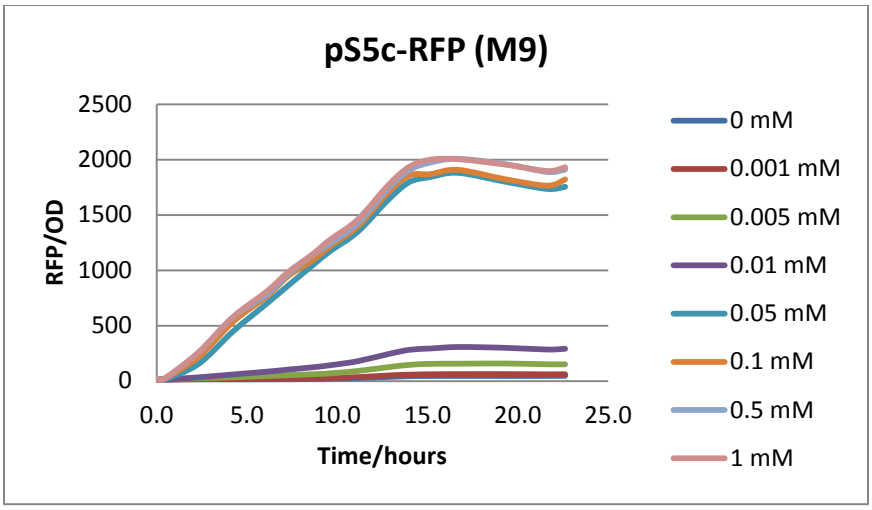


b

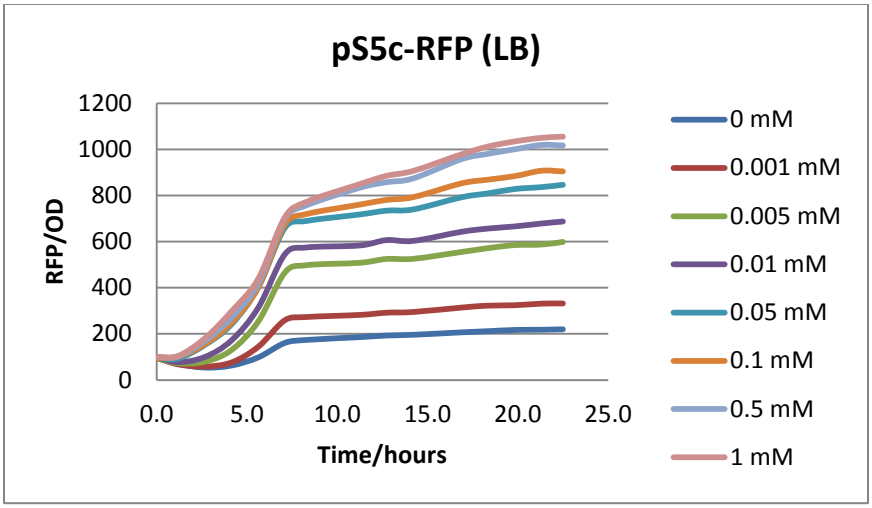


c

Figure 3.6 Results from the kinetic data of sensor responsiveness measurements in M9 medium. Strains are induced with different concentrations of IPTG. (a)(b). C2 strains are transformed with a vector carrying one LacUV5 controlled ZFP and a vector carrying synthetic promoter controlled reporter gene. (c). A repressor-free cell only transformed with a vector carrying synthetic promoter and its controlled reporter gene.



a

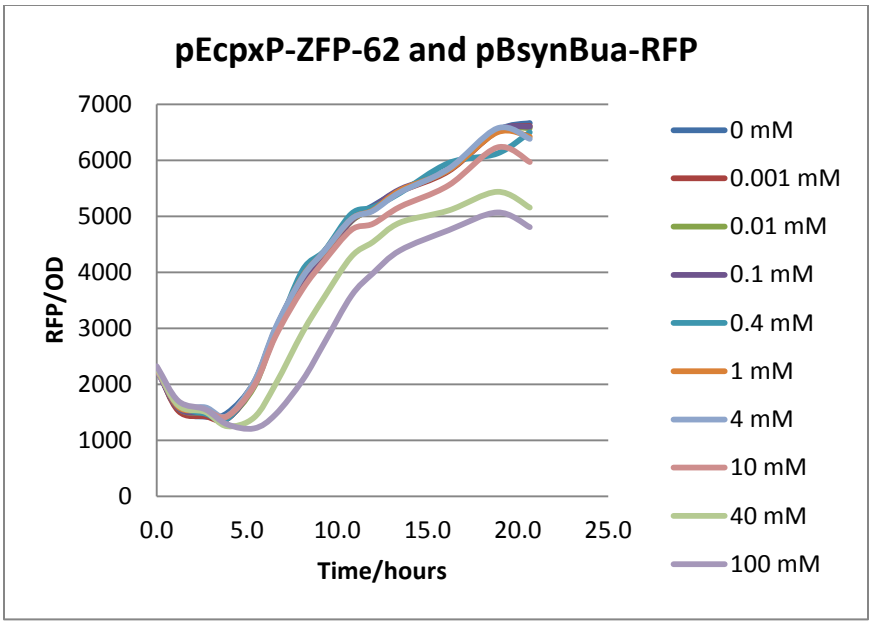


b

Figure 3.7 Result from the kinetic data of *LacUV5* IPTG titration. (a). *LacUV5* promoter titrated by IPTG in M9 medium. (b). *LacUV5* promoter titrated by IPTG in LB medium.

3.1.2.2 Native promoters

The native promoters were then integrated into our sensors. Though the sensor could not work as we expected due to the incapable of tuning, repression that correlated to the butanol-sensing promoters' properties could still be observed (Figure 3.8). The *metE* caused a stronger repression than the *cpxP* promoter. The *metE* caused a lower RFP fluorescence reading on both the initial value and the final value than the *cpxP*. Meanwhile, in the M9 medium, the ZFPs' repressing effect was also lost after 5 hours. In this experiment, the signal starts to increase after 5 hours. For the first 5 hours, the raw value of the fluorescence did not increase. The decrease on the figure was caused by cell dilution.



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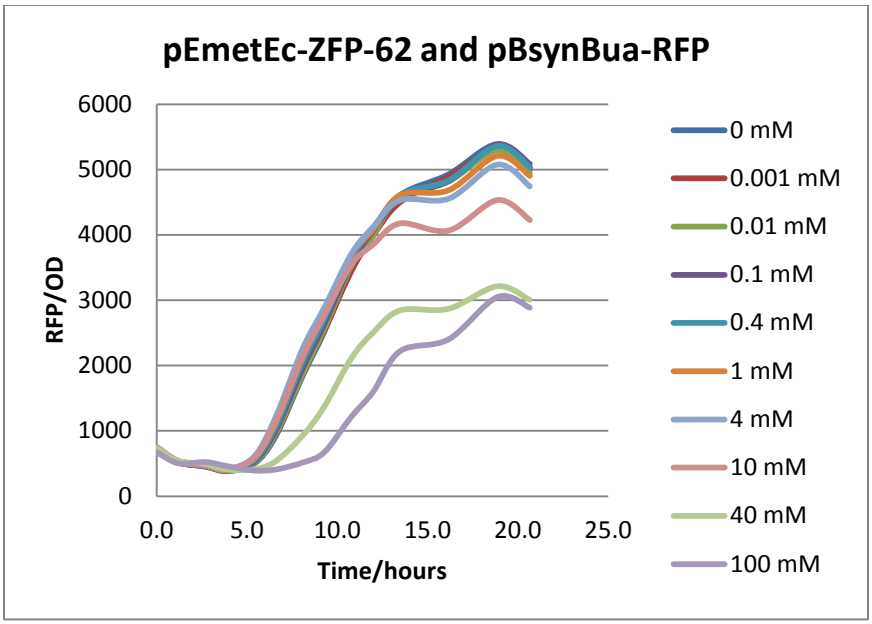


b

Figure 3.8 Results from the kinetic data of sensor responsiveness measurements in M9 medium with native promoters. Strains are induced with different concentrations of butanol. (a)(b). X2 strains are transformed with a vector carrying one native promoter controlled ZFP and a vector carrying synthetic promoter controlled reporter gene. (c). A repressor-free cell only transformed with a vector carrying synthetic promoter and its controlled reporter gene.

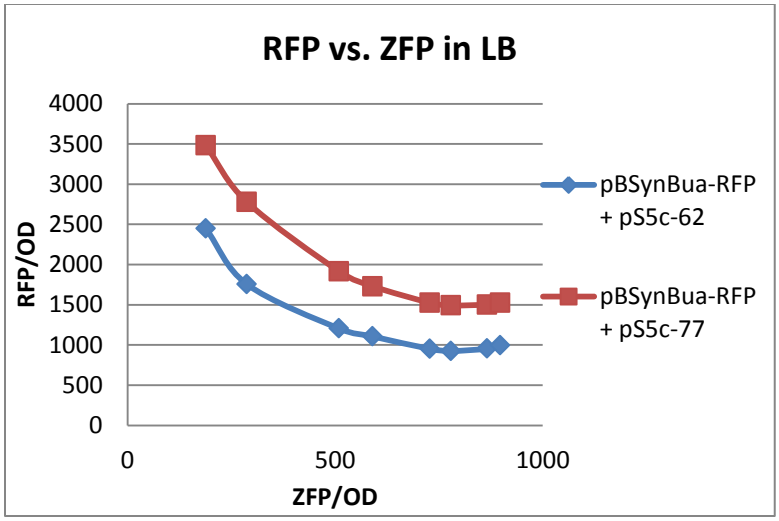
When treated with butanol, the growth of the cells with two sensor plasmids was heavily impeded. Compared with the X1 strain cells, a heavier arrested growth was observed in X2 strain when treated with the same amount of butanol. The massive production of RFP and ZFP could cause a heavy growth burden to the cells, and the cells' tolerance to butanol was decreased. This suggests that it is necessary to enrich the host's tolerance to butanol.

3.1.3 Sensitive Range

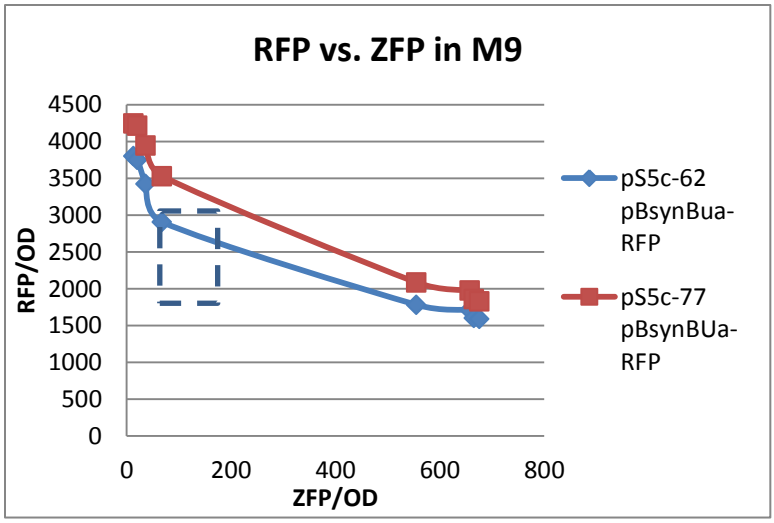
A butanol-sensing promoter's activity needs to change within a certain range in order to express appropriate amount of ZFP in order to make the sensor sensitive. By plotting the RFP value over the ZFP value, I was able to obtain the relationship of fluorescence level versus the ZFP level. The ZFP value is represented by the fluorescence value from the IPTG titration data of the pS5c-RFP promoter.

In LB medium, a comparatively sensitive region is when less than 600 arbitrary units of ZFP are expressed. For the M9 medium, in order to make the sensor more sensitive, no more than 200

arbitrary units of ZFP are needed for the first 5 hours. But some drawbacks will occur if low strength promoters are used. One of the drawbacks is the low signal-noise ratio and the sensor's specificity which is easily affected by the cells' natural regulations.



a

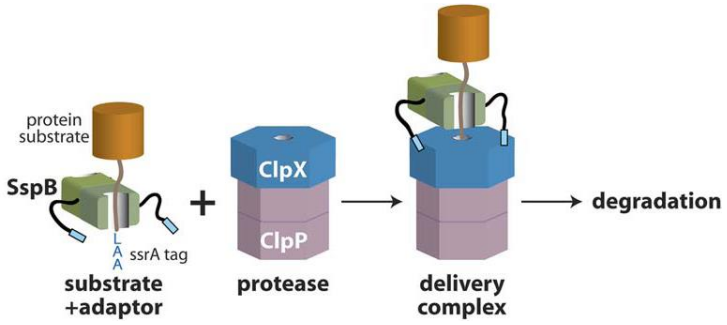


b

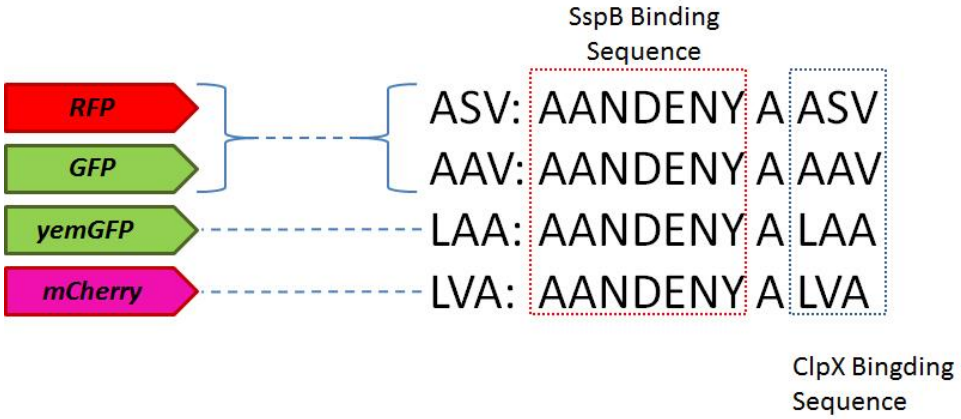
Figure 3.9 The relation between RFP expression level and ZFP level inside the sensor cells in LB medium and M9 medium

3.1.4 The Degradation Tag Studies

We next tested whether the C-terminal degradation tags could be used in our system to accelerate the degradation of the repressors. The dissociation constant of ZFP-synthetic promoter interaction is at the range of 10^{-8} M, which suggests a strong interaction between them. Our data also showed that the sensor only needs a low amount of ZFPs. In a negatively-regulated system, when native butanol sensitive promoter is repressed by butanol, the synthesis of the repressors stops. The existing repressors in the cell still need to be eliminated as soon as possible to make the sensor respond more rapidly. We used tagged fluorescent proteins to replace the regular fluorescent proteins in plasmid pS5c-RFP. The tags were the Ssr-A tags (Figure 3.10). The tags were fused to the C-terminus of the fluorescent proteins. When expressed in *E. coli*, the tagged-protein can be recognized by SspB and degraded by protease (Figure 3.10a) [31].



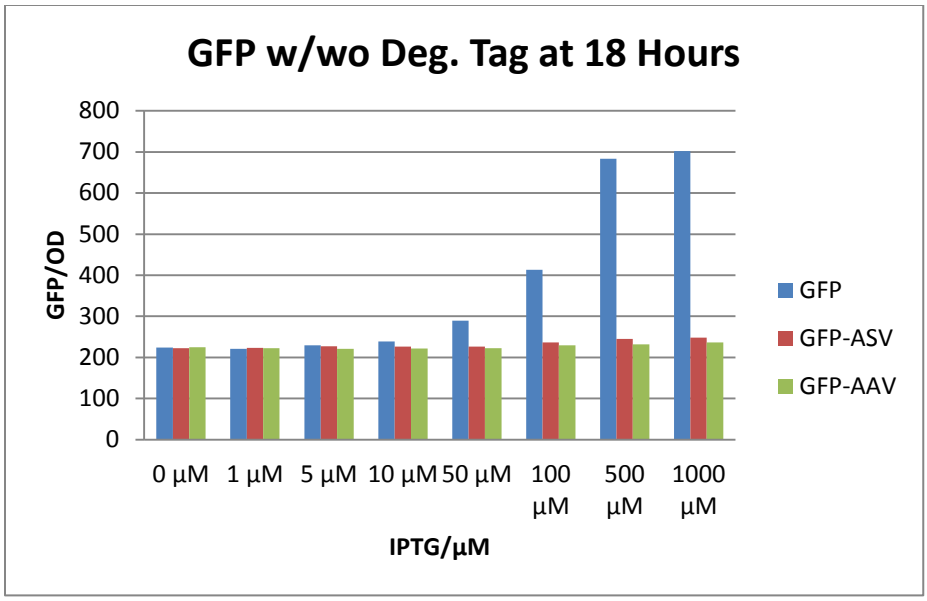
a



b

Figure 3.10 SsrA protein degradation tags. (a). example of SspB delivery of ssrA-tagged proteins to ClpXP [30]; (b) different tags were fused to several fluorescent proteins.

Two moderate strength tags, AAV and ASV [32], showed limited efficiency on RFP, but were more effective when coupled with GFP. The GFPs in the cell could be heavily degraded by AAV or ASV (Figure 3.11a), while the RFP level could only be lowered by small fraction (Figure 3.11b). This result indicates that the synthetic rate of GFP in the cell did not match the degradation rate, especially when low copy plasmid is used. The result also indicates that degradation tags are protein specified. Thus, for the ZFPs, a specific degradation tag for the ZFP family is necessary to be engineered. To do so, site-specific mutagenesis can be used and a high throughput screen method needs to be established.



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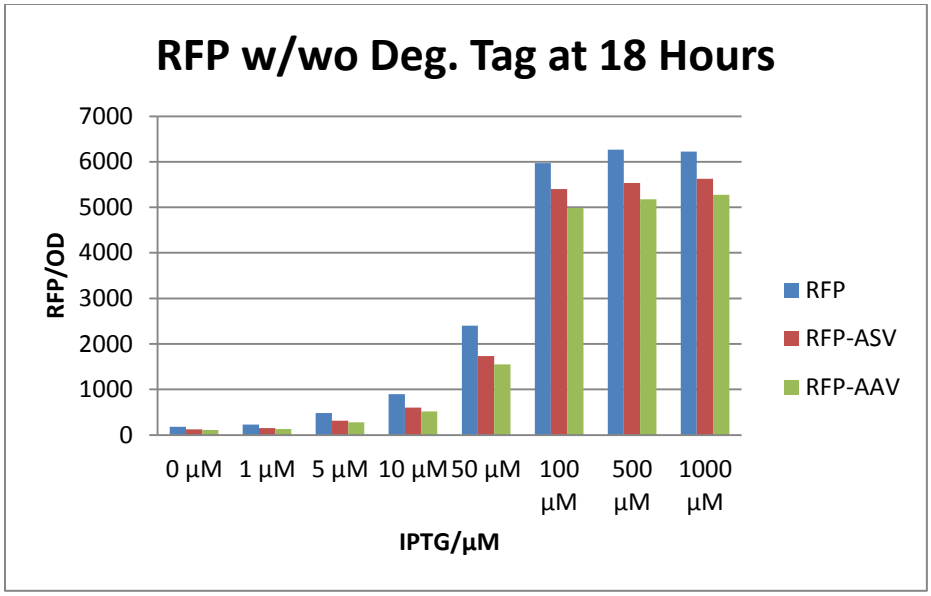
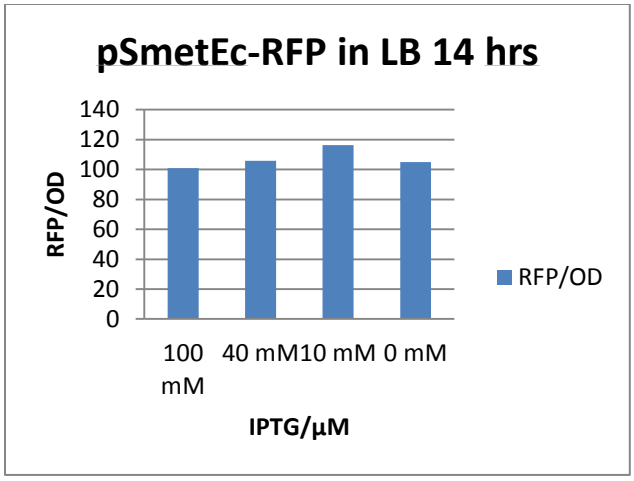


b

Figure 3.11 Results of degradation tag studies. (a). AAV and ASV tag on green fluorescent protein. (b). AAV and ASV tag on red fluorescent protein.

3.1.5 The Use of Growth Medium

In our study, many experiments were performed in both LB medium and M9. The dummy sensor experiment with IPTG titration had a better result in LB medium. Meanwhile, the RFP synthesis rate in M9 medium was also much higher than that in LB. This will cause a potential problem that the sensor will be less dose-sensitive in M9 medium. Even with very little de-repression, the RFP value will be very high. However, our experiment with native promoter can only be performed in M9 medium. The reason is that LB medium is incompatible with the *metE* promoter. We first discovered that the *metE* promoter appeared to be inactive in LB medium and have no response to butanol (Figure 3.12a). Further investigation showed that the *metE* is involved in the final step of the methionine biosynthesis in the absence of exogenously supplied vitamin B12 [33]. In the presence of the vitamin B12 cofactor, *metH* is functional and *metE* is repressed (Figure 3.13). In LB medium, vitamin B12 is present in the medium, and *metE* is repressed. In M9 medium, when no exogenous VB12 is supplied, the *metE* is activated to synthesize the methionine.



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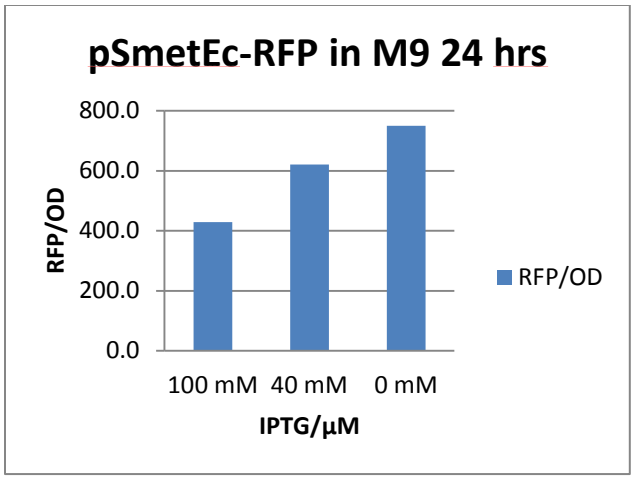

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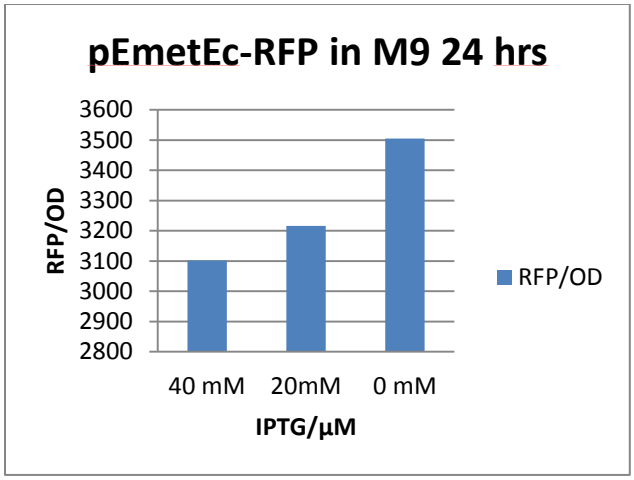

c

Figure 3.12 Promoter *metE* in different growth medium and induced with different concentration of butanol. (a) LB medium; (b) M9 Medium; (c) M9 medium.

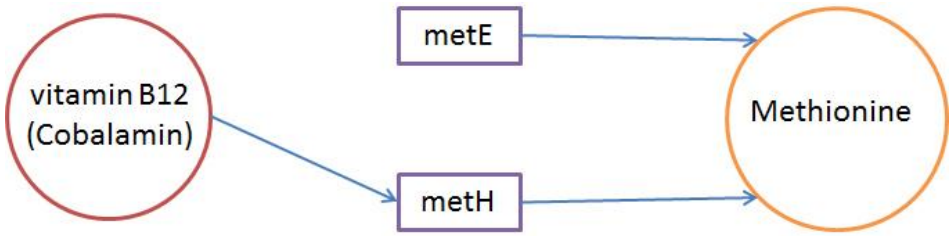

Figure 3.13 Final step of *de novo* methionine biosynthesis in *E. coli*.

3.1.6 *Clostridium* vs. *E. coli*

The butanol sensitive promoters we used were selected from the *E.coli* genomic DNA. *E. coli* doesn't have a native butanol related regulation pathway. In contrast, some native butanol production strains like *Clostridium acetobutylicum* [7] have many butanol related regulators. The response in *E. coli* is based on the cellular stress response, which is an indirect response to the butanol; while in *Clostridium*, we could find many butanol related regulators and butanol direct-response promoters, which may give us better specificity.

The reason we chose *E.coli* is because our final purpose is to establish a universal method for sensor design. We may find butanol-responding promoter in *Clostridium*, but we cannot find a one-substance-specified promoter for every chemical. The cellular stress response based methodology, however, could have the potential to create sensors response to any chemical.

3.2 Conclusion

This work has provided a preliminary study for the design of biosensors based on cellular stress response and transcription factors. For this type of sensors, identifying the promoters that are involved in a certain response is key. Once identified, fine tuning of those promoters will also be critical in order to increase the specificity of the sensor. Repression by the transcription factors involves many molecular interactions. The engineering of the zinc fingers and the synthetic promoter will have a major impact on the sensor's dynamic range.

This sensor design principle could provide a powerful screening tool for microbial biofuel production. It could establish a universal methodology for designing biosensor for any chemicals we wish to produce biologically. The biosensor can even be integrated into a fuel production strain as a self-indicator of productivity. The use of the biosensor can be extended to many other areas such as environment monitoring, clinical and food safe administrations.

Appendix A

Primers Used in This Study

Psynbu(F)	TTTTT GGTCTCAGGGAGAGTATAATGAGAGGGAAGGAGAGGAGTG GTG
Psynbu(R)	TTTTT GGTCTCATCCCACTATCTCCCATGTCAACAGCTCATTTCAGAAT ATTTG
PcysD(F)	TTTTT GACGTCTGAAACTGCCGCTGCGCTA
PcysD(R)	TTTTT GAATTCAACCGTTCCTTTGCAATAC
PgdhA(F)	TTTTT GACGTCAAAACTTTAGCGTCTGAGT
PgdhA(R)	TTTTT GAATTCAGATATAAAACCCTTATAT
Pompf(F)	TTTTT GACGTCTTTAGCGTCTTCAAGAGCC
Pompf(R)	TTTTT GAATTCTATTTATTACCCTCATGGT
PyagU(F)	TTTTT GACGTCACCAGGCCTCCGGTATTCT
PyagU(R)	TTTTT GAATTCATCTACCCTGCTTGTACCA
PaldB(F)	TTTTT GACGTCCGGTATTGGCGACTAATGCC
PaldB(R)	TTTTT GAATTCGATCGTCTCCTTGCGGTGTG
PargC(F)	TTTTT GACGTCTTGTTGACAC ACCTCTGGTC
PargC(R)	TTTTT GAATTCCGGGGCTATTCACCTTCTTAT
PcpxP(F)	TTTTT GACGTCTTGTTTAAATACCTCCGAGGCAG
PcpxP(R)	TTTTT GAATTCCATTTGCTCCCAAAATCTTTCTGT
PmetE(F)	TTTTT GACGTCGCCGCAGTTC CGCAACGCT
PmetE(R)	TTTTT GAATTCTTTTTCTTCCTCTAATTATATGTAAAT
PybcZ(F)	TTTTT GACGTCAAAATGACAA AATTGTCATT TTGCC
PybcZ(R)	TTTTT GAATTCATTTCCTCCG CATGTTGCCCG
PybjW(F)	TTTTT GACGTCTTTATTCTTAGCCTGTTAGTGCC
PybjW(R)	TTTTT GAATTCGATCACACCTTTTAAAGTTATATTTT
yemGFP(F)	TTTTT GAATTCAAAAGATCTGAGGAGAAAGGTACCATGTCTAAAG
yemGFP(R)	TTTTT CTCGAGTTTGGATCCTCACGCTGCAAGGGCGTAATTTTC

ASV_dbl_ter(F) TTTTT GGTCTCAAAAACTACGCTGCATCAGTTTAAGGATCCAAACTCG AGTAAGGATC

AAV_dbl_ter(R) TTTTT GGTCTCAAAAACTACGCTGCAGCAGTTTAAGGATCCAAACTC GAGTAAGGATC

RFP_ASV/AAV(F)

TTTTT CTCGGTATTTTCGTCGTTTGCTGCAGGCCTTTAAGCACCGGCG GAGTGACGAC

GFP_ASV/AAV(R)

TTTTT CTCTGGATTTTCGTCGTTTGCTGCAGGCCTTTTGTAGAGCTCA TCCATGCCATG

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