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Research Article

Improving the Lac system for synthetic biology

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Abstract. The *Escherichia coli lac* operon is controlled by a regulatory system that has been the subject of intensive study for the past fifty years. The system creates metabolic efficiency by responding to the levels of environmental lactose. In the absence of lactose, the LacI protein acts as a repressor of transcription from the *lac* promoter. Transcription begins when lactose binds to LacI, which results in the expression of three genes involved in lactose uptake and catabolism. The *lac* promoter is the most commonly used promoter in the field of synthetic biology. Although it is widely used, the *lac* promoter is known to have leaky transcription, meaning that transcription takes place even when the repressor is present and the inducer is absent. In an effort to redesign the *lac* promoter, we tested pLac variants that were reported to have a higher affinity for RNA polymerase than the wild-type. We also compared three mutants of the LacI repressor that were reported to have increasing affinity for the pLac promoter. Using GFP reporter constructs, we found that the pLacI^{Q1} promoter showed much higher levels of transcription than the wild-type promoter. Of the twelve combinations of promoters and repressors tested in the presence and absence of an inducer, we discovered that the wild-type LacI repressor protein with the pLacI^{Q1} mutant promoter is the best combination for high levels of induction and low levels of leaky transcription. Our results promise to help synthetic biologists design and build systems with tighter regulatory control.

Introduction

Synthetic biology is a new field of study that blends biology with mathematics, computer science and engineering. Synthetic biologists are designing and building DNA devices to alter the output of cells for important applications in medicine, energy, technology, and the environment (Baker et al., 2006). For example, synthetic biologists in Scotland designed and constructed bacteria that can visually warn people about trace amounts of arsenic in drinking water (University of Edinburgh, 2006). Other synthetic biologists at UC Berkeley reengineered microbial metabolism to produce a powerful anti-malaria medication for one tenth the cost of conventional production methods (Ro et al., 2006). Using bacteria to perform such functions is beneficial and may soon lead to the production of carbon-neutral biofuels from waste material (Service, 2008). In order to build systems that can efficiently produce desired outputs (like those described above), tight control of genetic circuits is required to turn the system

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on or off in response to specific environmental cues. If a regulatory system does not function properly, the entire synthetic device could malfunction. Our goal was to improve the leaky transcription of the most widely used regulatory system in the field of synthetic biology: the *lac* system.

The *lac* operon in *Escherichia coli* consists of a transcriptional promoter, an operator that binds a repressor, and three structural genes (Gilbert and Maxam, 1973). The structural genes code for proteins that are needed only when lactose is available for catabolism. In the absence of lactose, the LacI protein acts as a repressor of the pLac promoter by binding to the operator and blocking transcription of the operon (Lewis et al., 1996). It is important to note that the vast majority of previous research with the *lac* operon addresses the chromosomal *lac* operon, and not the engineered components on high-copy plasmids used in our study, though the two are closely related. One of the major differences between the chromosomal pLac and the engineered pLac is that the operator of the operon is incomplete in the plasmid version of pLac (Dickson et al., 1975). The entire *lac* operon has three operator repressor recognition sites and all three of these recognition sites are required for complete repression (Lewis et al., 1996). Leaky transcription is particularly evident when fewer than all three operator sites are used. Induction of the *lac* operon occurs when the repressor adopts a structure which prevents operator binding after LacI binds either lactose or a synthetic inducer such as the lactose analog isopropyl-β,D-thiogalactoside (IPTG; Glascock and Weickert, 1998). De-repression allows transcription of the genes needed for lactose uptake and catabolism.

The widely used engineered version of the pLac promoter retains only one LacI binding site which probably contributes to the leaky transcription in the plasmid version of this system. In addition, when the repressor binds to the promoter, the chromosomal version forms a DNA loop that prevents transcription (Lewis et al., 1996). The plasmid copy does not form this loop as efficiently, which could contribute to incomplete repression (Setty et al., 2003). The engineered pLac system is about five times noisier than the comparable *recA* promoter, which means pLac transcription varies between individual cells within a population (Kuang et al., 2004). This variation between cells is partially due to the fact that the *lac* induction mechanism requires the function of multiple proteins, meaning that different cells have different rates of initiating transcription.

We investigated the efficiency of transcription and repression of mutant pLac promoters and LacI repressors in order to see which combinations would produce a system with the largest dynamic range of transcription. The *lac* promoters we built and tested were wild-type pLacI, $pLacI^{Q}$ and $pLacI^{Q1}$ (Glascock and Weickert, 1998; Table 1). The promoters were reported to have different affinities for RNA polymerase, with pLac having the lowest and $pLacI^{Q1}$ having the highest ("Q" portion of the lac promoter nomenclature denotes a constitutive mutation; Global Energy Group, 2010). $pLacI^{Q}$ has a point mutation in the -35 region of the promoter in which a G is replaced by a T. $pLacI^{Q1}$ has a 15 bp deletion from the $pLacI^{Q}$ promoter and has an altered -35 region. Glascock and Werickert demonstrated that $pLacI^{Q1}$ increased the strength of the promoter 170-fold and it is 17-fold stronger than $pLacI^{Q}$. We also investigated four different repressor proteins: wild-type LacI, LacI-I12, LacI-X86 and LacI-I12/X86. The LacI variants have missense mutations that confer an increasing affinity for the repressor (Schmitz and Galas, 1980). The LacI-I12 protein has a missense mutation at amino acid three, which changes proline to tyrosine (3P→Y). LacI-X86 changes amino acid sixty-one from serine to tyrosine (61S→Y). Both of these mutations cause a 50 to 100 fold increase in binding affinity of the repressor for operator DNA. The LacI-I12/

Table 1. Promoters and repressors. This table shows all the different promoter and repressor mutations used in the study.

Promoters	Repressors
LacI	LacI
$LacI^{Q}$	LacI-I12
$LacI^{Q1}$	LacI-X86
	LacI-I12/X86

X86 double mutation has a 10,000 fold increase in binding affinity for the operator (Schmitz and Galas, 1980). We constructed each of the promoters and repressors as BioBrick parts and submitted them to the Registry of Standard Biological Parts (Knight, 2003). We tested all twelve combinations of the three promoters and four repressors and quantified transcriptional output using GFP fluorescence. Our goal was to provide the synthetic biology community with a *lac* system that enables better control of genetic circuits.

Materials and Methods

Construction of inserts

Some components were obtained from the Registry of Standard Biological Parts (http://partsregistry.org/Main_Page). Parts not in the registry were synthesized using PCR or dsDNA oligo assembly. For primer-dimer dsDNA assembly using PCR, the following procedure was used: 1) Denaturation at 95°C for 5 min. 2) 95°C for 30 sec. 3) Anneal at 5°C lower than T_m (found at http://www.promega.com/biomath/calc11.htm; salt concentration adjustment was set at "Promega Master Mix") 4) Elongation at 72°C at one minute per kb 5) Repeat cycle from step two 29x 6) Hold at 25°C. The dsDNA oligo assembly protocol can be found online. Lancelator (http://gcat.davidson.edu/IGEM06/oligo.html) was used to determine oligo sequences with similar melting points.

Verification and purification of insert

All enzymes used in digestions were used as per the manufacturer's (Promega) instructions. Enzymes regularly used included EcoRI, PstI, XbaI, SpeI, and MluI. Gel electrophoresis was performed in 0.5X TBE at 100V and 400mA. The optimal percent gel for a particular sized DNA fragment was determined using the gel optimization tool (http://gcat.davidson.edu/iGEM08/gelwebsite/gelwebsite.html). If electrophoresis was not sufficient to determine whether or not the insert was correct, inserts were sent off for sequencing at Clemson University in the form of 90ng of dried down DNA. Inserts were purified using a gel purification kit (Qiagen, Catalog no. 28706).

Cloning

Enzymes used in ligations were used following the manufacturer's instructions (Promega). Plasmids were transformed into JM109 strain *E. coli* cells using either Zyppy or heat-shock transformation and plated on LB media containing the desired antibiotic. Plasmids were purified using Promega plasmid purification kit (Catalog n:. A1460). DNA concentration was determined using a nano-drop (ND-1000 spectrophotometer).

Screening for successful ligations

Used colony PCR and followed the same protocol as for primer-dimer dsDNA assembly except time span of elongation step is modified based on the length of the insert; 1 minute per kb. The cycle was repeated 19 times instead of 29 times. Ingredients of PCR mixture for colony PCR can be found online at http://gcat.davidson.edu/GcatWiki/index.php/Davidson_Missouri_W/Davidson_Protocols.

Designing pLac and LacI

The first step in this procedure was to design and build the three *lac* promoters (pLacI, pLacI^{Q}, pLacI^{Q1}) and the four LacI proteins (LacI, LacI-I12, LacI-X86, LacI-I12/X86; Figure 1). The sequences for all the proteins and promoters can be found in the Registry of Standard Biological Parts (http://partsregistry.org/Catalog). Initially, we performed primer-dimer PCR for the construction of all the promoters and proteins

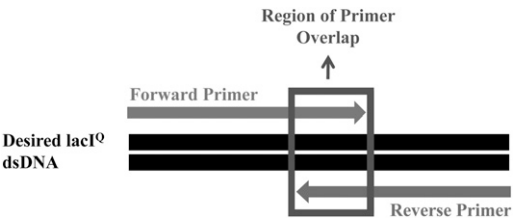

Figure 1. Designing primers for the construction of the lac promoters using primer-dimer method. This method is useful for synthesizing DNA sequences that are relatively short (less than 150 bp). Primers anneal for at least 12bp overlap between the two primers. DNA polymerase elongates the rest of the sequence to produce double-stranded DNA.

and were able to successfully build wild-type pLac promoter and pLacI^{Q} promoter. For pLacI^{Q1}, the primer-dimer method produced many mutations in the sequence and we had to use a different method, called oligo assembly, to build this promote (Figure 2). In this method, the oligonucleotides self assemble into double-stranded DNA. The sequences were designed using an online tool created by a previous iGEM team (http://gcat.davidson.edu/IGEM06/oligo.html).

For the LacI variants, we used PCR to amplify genomic DNA (*E. coli* strain MG1655) to build the entire protein. We successfully amplified wild-type LacI and LacI-I12. For LacI-X86 and LacI-I12/X86, we tried several methods to build the coding DNA including a two-round PCR approach, site-directed mutagenesis and a modified site-directed mutagenesis procedure. All of these attempts failed, so we had portions of the variants synthesized by GeneArt (http://www.geneart.com/). For this approach we found unique restrictions sites (XbaI and MluI) that flanked the site of the X86 and I12 mutations (Figure 3). We had these segments synthesized and then ligated them into the appropriate plasmids previously cut with XbaI and MluI.

Testing Constructs

In order to build the test constructs, we used a combination of parts from the Registry of Standard Biological Parts and parts we constructed. We used the BioBrick assembly method, developed by Tom Knight, to assemble these constructs (2003). This standardized assembly method uses common restriction sites that flank every part in this database, so that all parts within the registry can be ligated together. This allows the user to build a variety of different constructs with the desired output.

In order to test the constructs, we transformed the twelve final constructs into HB101 cells which lack the LacI gene so that the testing constructs were the only source of repressor molecules. As controls for this round of testing, we transformed the testing constructs lacking LacI into HB101 cells in order to measure default level of transcription for each promoter variant.

Measuring construct output

We used a fluorometer (BioTek, FLx800, Excitation=485/20, Emission=528/20) and a spectrophotometer (BioTek, ELx808, OD 595). For both the *lac* promoter control constructs, we used two independent colonies and allowed 16 hours of overnight growth. For the promoter-repressor constructs, we followed the same procedure mentioned above, but also measured fluorescence in the presence or absence of 3μg/mL IPTG. Because IPTG affects cell growth and GFP fluorescence, the promoter-repressor construct data were further normalized to the expression levels of pLacI^{Q1} which was set to a value of 1 to permit comparisons between the different experimental conditions.

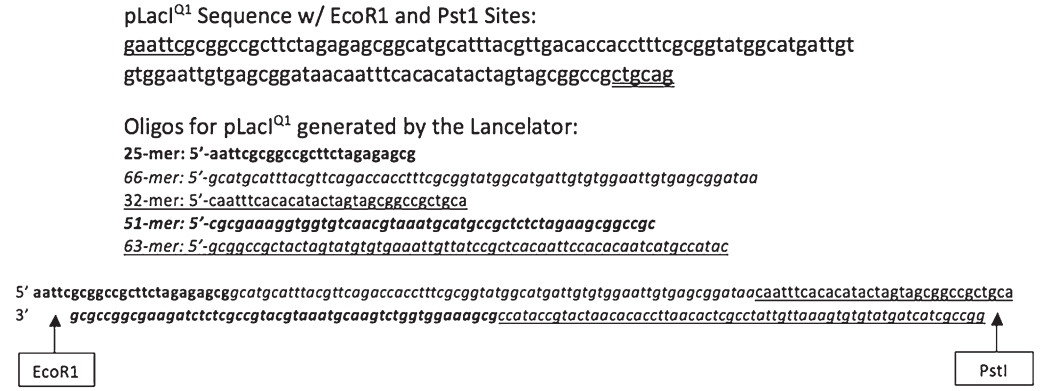

Figure 2. Building the LacI^{Q1} promoter using oligo assembly. The LacI^{Q1} promoter sequence was flanked by BioBrick ends including EcoRI and PstI sticky ends so that it would be easy to ligate this part into a plasmid. The EcoRI site is underlined and the PstI site is double-underlined in the original sequence. The Lancelator website produces oligonucleotide sequences with very similar melting points.

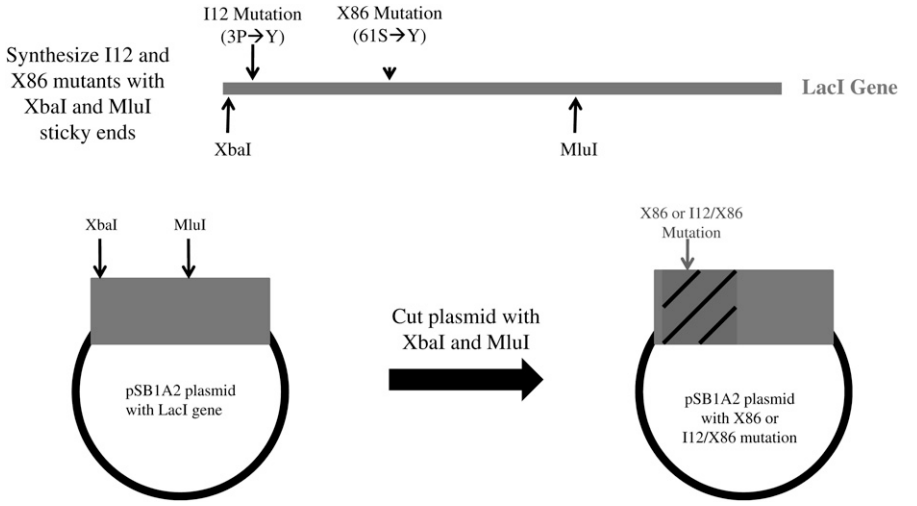

Figure 3. Mapping restriction sites for producing LacI-X86 and LacI-I12/X86. LacI wild-type plasmid was cut using XbaI and MluI so that the synthesied LacI-X86 and LacI-I12/X86 DNA fragments could be inserted into this plasmid. The red color indicates the LacI gene and hashed blue region indicates the region where either the X86 or I12-X86 mutation was inserted.

Results

We designed and constructed two mutant versions of pLac as BioBrick compatible parts, verified them by DNA sequencing, and submitted them to the Registry of Standard Biological Parts (Table 1). We wanted to validate that the mutant forms of the *lac* promoter functioned as predicted by Glascock and Weickert (Glascock and Weikert, 1998). We cloned the wild-type and mutant pLac promoters upstream of a GFP reporter construct and measured the output of GFP using fluorescence. Figure 4 shows the normalized fluorescence measurements for the three pLac variants. The output of the pLacI^{Q} mutant promoter was 6-7 times greater than the wild type promoter, and the pLacI^{Q1} mutant was about 50 times stronger than the wild-type which is consistent with our expectations (Glascock and Weickert, 1998).

We also designed and cloned three mutant variants of the LacI repressor as BioBrick compatible parts (Table 1). We inserted four LacI variants upstream of the three plac-GFP reporter constructs (Figure 5). These constructs enabled us to test the ability of the four LacI repressor variants to repress each of the three *lac* promoters in the presence and absence of the IPTG inducer (Figure 6). To compensate for the negative growth effects of IPTG on half of the samples, all of the data were normalized to the expression levels of pLacI^{Q1} in the absence of repressor to facilitate direct comparisons. In the absence of a repressor, the relative levels of expression from the three promoters were as expected, and none of them were induced by IPTG (Figure 6). Figure 6 also shows the effects of each repressor on all three promoters. In the absence of the IPTG inducer, each repressor should down regulate the expression driven by each promoter. Of the twelve combinations of promoters and repressors, only four showed substantial dynamic ranges (LacI+pLacI^{Q1} > LacI-I12+pLacI^{Q} > LacI+pLac > LacI+pLacI^{Q}; Figure 6).

In our synthetic *lac* system, dynamic range refers to GFP production in the presence or absence of the IPTG inducer. Although all twelve combinations of promoters produced GFP, the LacI+pLacI^{Q1} construct shows the largest dynamic range (Figure 6). In the presence of IPTG, the LacI+pLacI^{Q1} construct produced more GFP that LacI+pLac or LacI+pLacI^{Q}, because the pLacI^{Q1} is the strongest promoter. The only LacI-I12 construct that demonstrated the desired behavior was LacI-I12+pLacI^{Q}. Induction by IPTG was better when LacI-I12 was paired with pLacI^{Q} than pLac because placI^{Q} is a stronger promoter. However, GFP production in the absence

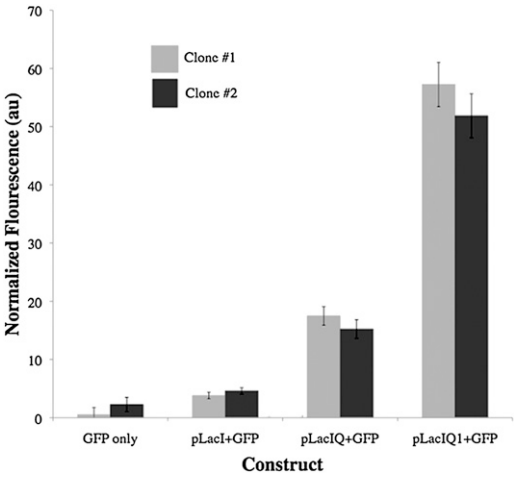

Figure 4. Characterization of Lac Promoters. Fluorescence divided by optical density of the three lac promoter + GFP constructs. RBS-GFP with no promoter served as the control. Error bars represent the standard deviation of the normalized fluorescence.

of IPTG was repressed better when Lac-I12 was paired with pLac. This difference may be useful depending on whether the desired output is stronger repression or greater induction. In the presence of IPTG, only Lac-I12+pLacI^{Q} exhibited substantial induction compared to the LacI constructs.

In comparison to the LacI and LacI-I12 repressor variants, LacI-X86 and LacI-I12/X86 repressors were not inducible by IPTG, except for LacI-X86+pLacI^{Q1} which exhibited moderate induction (Figure 6). Contrary to our expectations, constructs containing X86 alone or in combination with I12 exhibited undesirable

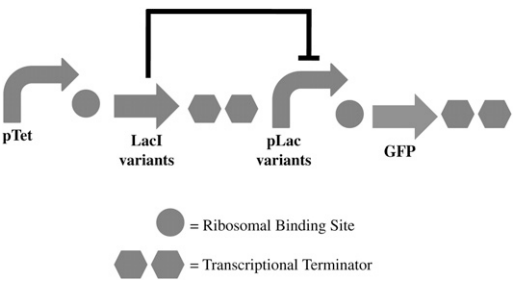

Figure 5. Testing construct for the three promoters with the four proteins. Generic design for the various lac promoters and proteins. The constitutive pTet promoter activates the transcription of the LacI variants which repress pLac variants and GFP. The circles are ribosomal binding sites (RBS) and the double hexagons are transcriptional terminators.

properties. For the LacI-X86 and LacI-I12X86 constructs, the level of expression in the absence of IPTG was higher than the respective promoter-only controls, except in the constructs where pLacI^{Q1} was the promoter (Figure 6). In the presence of IPTG, LacI-X86+pLacI^{Q1} and LacI-I12/X86+pLacI^{Q1} had lower levels of GFP expression than their respective controls. Furthermore, the LacI-X86 and LacI-I12/X86 constructs showed much higher levels of sample to sample variation in the absence of IPTG than LacI and LacI-I12. Overall, the LacI+pLacI^{Q1} construct produced the largest dynamic range between induction and repression.

Discussion

By deconstructing and redesigning the *lac* operon, we gained novel insights into the use of the *lac* system in synthetic biology. In some cases, the data met our expectations, but our experiments also produced some unexpected results. We had predicted that the LacI-I12/X86+pLacI^{Q1} would produce the largest dynamic range, but we found that the LacI+pLacI^{Q1} construct most closely models the desired behavior (Figure 6). In the absence of IPTG, GFP production was as low as the wild-type LacI/pLac and in the presence of IPTG, the expression levels were greatly increased though we observed sample to sample variation. As previously noted, the *lac* system has a complex induction mechanism, meaning that different cells may have different rates of induction which was most obvious in the LacI+pLacI construct (Kuang et al., 2004). The new combination of the pLacI^{Q1} and LacI greatly reduces the noise caused by the different rates of induction (Figure 6).

The LacI-I12 constructs seemed to function in agreement with previous research. Overall, constructs with LacI-I12 repressed the promoters more than the LacI. However, none of the LacI-I12 constructs were induced as well as LacI when exposed to IPTG. Schmitz and Galas (1980) documented that LacI-I12 has a 50–100 fold increase in binding affinity for the operator. The LacI-I12+pLacI^{Q1} construct demonstrated an increased affinity because this construct had lower GFP expression levels than that of the

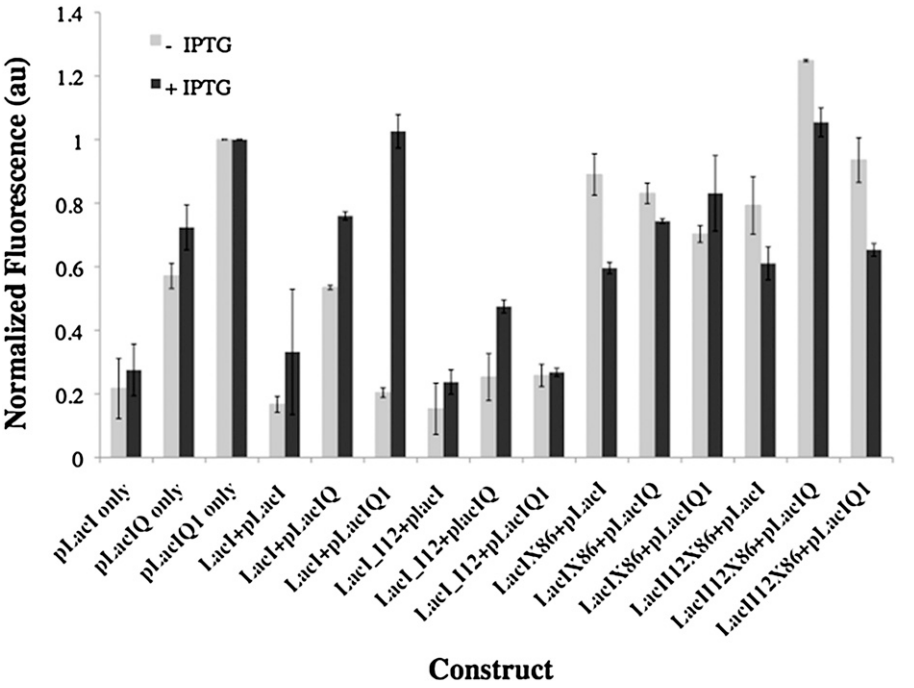

Figure 6. Characterization of LacI, LacI-I12, LacI_X86 and LacI_I12_X86 proteins. Flourescence divided by optical density expression levels of pLacIQ1 in the presence and absence of IPTG were set to 1 as standards. All other constructs were normalized to the appropriate standard to facilitate comparisons between the different constructs. Error bars represent the standard deviation.

control in both the presence and absence of IPTG. It is possible that the higher affinity of the LacI-I12 protein caused this protein to resist dissociation when exposed to IPTG which might indicate LacI-I12 alters the manner in which IPTG binds to the repressor. LacI-I12 also has higher binding affinity for non-operator DNA (Schmitz and Galas 1980). All of our constructs were tested in high copy number plasmids, which might have produced behavior that was different from that of the single copy *lac* operon on the *E. coli* chromosome. The ability of LacI-I12 to bind to non-operator DNA combined with the large number of plasmids inside of each cell presented the repressor with a large number of non-specific binding sites, which could have contributed to the noise seen in the constructs with this protein as the repressor.

The results for the LacI-X86 and LacI-I12/X86 constructs showed some interesting differences with previous research. LacI-X86 was reported to have a 50-100 fold increased affinity for the repressor and LacI-I12/X86 was measured to have a 10,000 fold increased repressor affinity (Schmitz and Galas 1980). From these previous results, we had predicted that the LacI-X86 repressor would have reduced expression of all promoters more than the wild-type LacI repressor. However, Figure 6 shows LacI-X86 and LacI-I12/X86 had higher levels of expression compared to LacI and LacI-12 constructs (Figure 6). Since LacI-X86 and LacI-I12/X86 repressors have not been previously tested with pLac promoter variants, we can only speculate about possible reasons for these results. It could be that the LacI-X86 mutation reduces the ability of repressors to bind to the operator and to respond to induction by IPTG. This could be a plausible explanation for two reasons: 1) all except one construct (LacI_X86+pLacI^{Q1}) has a higher level of expression in the absence of IPTG and 2) mutant promoters and repressors could alter their interactions with each other and remove the need for induction by IPTG.

All of our pLac variants were truncated compared to the promoter found in the native operon. In order for repression to be complete, all three operator sites in the chromosomal version of pLac must be present (Lewis et al., 1996). These operator sequences are located throughout the *lac* operon, with one of them located downstream of the *lacZ* gene which is missing from our pLac variants. LacI binds the three operator sequences together and results in the formation of a DNA loop, which effectively blocks transcription. Thus, our truncated pLac variants not only lack two binding sites but the formation of a stable loop complex may not be possible because of the shorter length of the DNA. The decreased ability of the truncated promoter to form a stable DNA loop combined with the mutations in the LacI-I12, LacI-X86 and LacI-I12/X86 could produce a novel induction mechanism for the *lac* system. GFP expression levels were unaffected by IPTG in X86-containing repressors and LacI-I12+pLacI^{Q1}. Because complete repression is not possible in the absence of all three operator sequences, some other mechanism may also be at work here, such as induction by the *lac* proteins. Another possible explanation could be that LacI-I12, LacI-X86 and LacI-I12/X86 are able to bind to operator DNA, but are not able to derepress. These repressor variants have been shown incapable of transforming the DNA into a completely repressed state, called the allosteric transition (Lewis et al., 1996). These two hypotheses, the repressors functioning as inducers and the inability of LacI variants to repress, provide possible explanations for why some constructs have higher levels of expression in the absence of IPTG.

In the future, it would be interesting to design, construct, and test three-operator versions of pLac promoters with LacI repressor variants to see how the increased number of operator sites would affect regulation. It would also be interesting to test our constructs in the presence of varying concentrations of IPTG to experimentally determine whether or not LacI variants require higher concentrations of IPTG. These experiments would shed light on the natural function of the *lac* operon and increase the toolkit of regulatory elements that could be used by synthetic biologists to design and build genetic circuits.

Synthetic biology is a growing field that combines biology, mathematics, computer science and engineering (Campbell et al., 2006). Its practitioners use genetic circuits to engineer cells to carry out new functions with important applications. Their work requires predictable regulatory control of gene expression. Our research in characterizing the transcriptional outputs of twelve different combinations of *lac* promoters and repressors has yielded results that will be of broad interest to the synthetic biology community.

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