Transcriptional control of gene expression with oxygen sensitive promoters in *Escherichia Coli*

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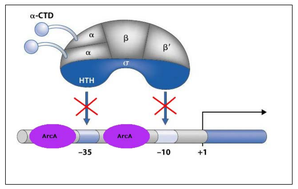
Introduction

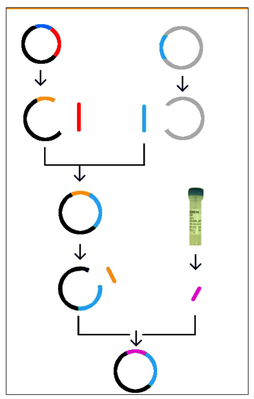
Synthetic biology is an emerging field which attempts to re-design and control natural biological systems for useful purposes. Nucleotide sequences encoding genes, promoters, and terminators can be freely interchanged within a genome. By creating a registry of genetic parts, researchers can combine an array of parts to create a desirable biological system. In most cases, this system alters the organism's natural metabolism to create a more valuable product (metabolite) from raw feedstock.

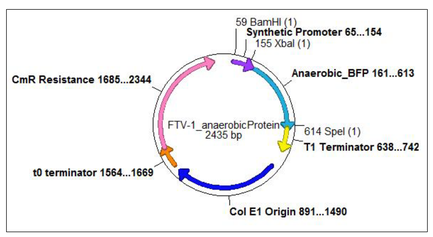
The main objective of this project was to create a series of oxygen sensitive promoters to repress gene transcription. Repressing promoters offer a convenient way to control the protein production rate of competing reactions in a metabolic pathway. As an example in biofuel production[1], non-native butanol can be produced in bacteria by reducing glucose to pyruvate to 2-ketoisovalerate to isobutanol. Competing, undesirable metabolic paths that utilize glucose as an energy source can be slowed or halted by repressing enzyme production. In most natural promoters, ArcA acts a a transcriptional repressor. Therefore, this project focused on its ability to repress transcription. ArcA is actually one part of a two-component ArcA / ArcB system. Under low oxygen or completely anaerobic conditions, the membrane bound ArcB sensor kinase autophosphorlyates and transphosphorylates the ArcA response regulator. ArcA-P can bind to the promoter region of a gene to repress transcription. The ArcA consensus binding sequence was used and its location on the promoter varied to compare the transcription rate to a constitutive promoter.



Methods

Escherichia Coli Flexible Test Vector 1 was used as the plasmid backbone. The built-in red fluorescent protein reporter gene requires oxygen to fluoresce, and thus was inappropriate for this problem. We ordered a predesigned blue fluorescent protein (excitation: 450nm, emission: 495nm) that would fluoresce without the presence of oxygen. By performing a restriction digestion upon both plasmids with XbaI and SpeI enzymes, the red fluorescent reporter gene was replaced in an ensuing ligation. The synthetic promoters were designed using the software A Plasmid Editor and purchased from Integrated DNA Technologies. Each promoter was assembled using PCR. The native FTV1 sigma 70 constitutive promoter was removed by performing another restriction digestion with XbaI and BamHI enzymes. The synthetic promoters were then ligated into the E. coli backbone to complete the new plasmid.





Experimental Design

The consensus and natural RNA polymerase -10 and -35 hexamer sequences (shown in red and blue) were used along with the consensus ArcA binding sequence (shown in purple) to repress transcription. To achieve the strongest repression, the location of the ArcA binding sequence was varied based on natural promoter sequences.

Natural Promoter 1
ATATGGATCCCTTTACTCCTTGTTGTCGCGGTGGTCTGCGGATTAAGCGCAAATAGCGTTTGCTGTGTTAATTAAATGTTAAATAAACTAGGTGTGACGTTCTAGATATA
-35 -10

Consensus Promoter 1
ATATGGATCCCTTTACTCCTTGTTGTCGCGGTGGTCTGCGGATTAAGCGCAAATAGCTTGACATGTGTTAATTAAATGTTAATATAATTAGGTGTGACGTTCTAGATATA
-35 -10

Natural Promoter 2
ATATGGATCCCTTTACTCCTTGTTGTCGCGGTGGTCTGCGTTAATTAAATGTTAAGCGTTTGCTGTGTTAATTAAATGTTAAATAAACTAGGTGTGACGTTCTAGATATA
-35 -10

Consensus Promoter 2
ATATGGATCCCTTTACTCCTTGTTGTCGCGGTGGTCTGCGTTAATTAAATGTTAAGCTTGACATGTGTTAATTAAATGTTAATATAATTAGGTGTGACGTTCTAGATATA
-35 -10

Natural Promoter 3
ATATGGATCCCTTTACTCCTTGTTGTCGCGGTGGTCTGCGTTAATTAAATGTTAAGCGTTTGCCGAATCGAGGATCGGCAAGATAAACTAGGTGTGACGTTCTAGATATA
-35 -10

Consensus Promoter 3
ATATGGATCCCTTTACTCCTTGTTGTCGCGGTGGTCTGCGTTAATTAAATGTTAAGCTTGACACGAATCGAGGATCGGCAAGTATAATTAGGTGTGACGTTCTAGATATA
-35 -10

Consensus Promoter 4
ATATGGATCCATGGTGTTAATTAAATGTTACTTACTCCTTGTTGTCGCGGTGGTCTGCGTTAATTAAATGTTAAGCTTGACACGAATCGAGGATCGGCAAGTATAATTAGGTGTGACGTTCTAGATATA
-35 -10

Consensus Promoter 5
ATATGGATCCATGGTGTTAATTAAATGTTACTTACTCCTTGTTGTCGCGGTGGTCTGCGTTAATTAAATGTTAAGCTTGACATGTGTTAATTAAATGTTAATATAATTAGGTGTGACGTTCTAGATATA
-35 -10

Oxygen Gradients

Since ArcA is phosphorlyated over a range of oxygen conditions[2], we must test its repressing effectiveness over a range of oxygen conditions. By suspending *E. coli* cells vertically in an agarose gel, we can create an oxygen gradient in which oxygen diffuses downwards through the agarose gel and is consumed by each strata of cells. To independently and qualitatively asses the oxygen content a redox indicator dye, resazurin, was used. As shown below, a sharp oxygen gradient was produced. In order to create an appreciable oxygen gradient, three variables needed to be simultaneously optimized: agarose gel percentage, dye concentration, and number of cells.

To describe the oxygen content with respect to depth quantitatively, a Green's function was used. This model provides the oxygen content with respect to depth, which we can relate to fluorescence measurements. By approximating the diffusion flux with the oxygen concentration, we can rationally refine our agarose percentage and cell count to produce a more gradual gradient than shown in the photo below.

$$\frac{dC_{O_2}}{dt} = D\frac{d^2C_{O_2}}{dz^2} - kC_{O_2}$$

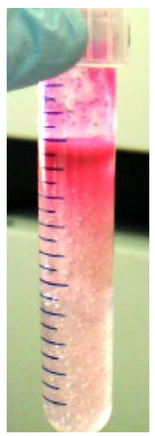
$$C_{O_2} = p\frac{\sinh\left(\frac{(L-x)\sqrt{D\cdot k}}{D}\right)}{\sinh\left(\frac{L\sqrt{D\cdot k}}{D}\right)}$$

At steady state

$$q = -D\frac{dC}{dz}$$

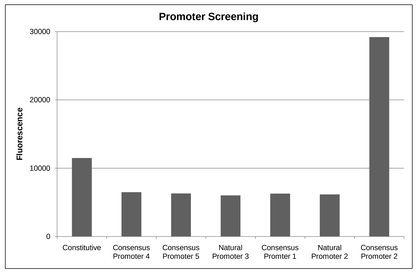
$$q \approx -D\frac{\Delta C_{O_2}}{L}$$

p = Oxygen concentration at surface (partial pressure)
D = Diffusivity of O2 in agarose+LB mixture
L = Sample depth
k = Oxygen consumption rate of cells per unit volume



Preliminary Results

Preliminary results based on a fluorescence screening suggest that 6 of the 8 synthetic promoters may have properly ligated and affected transcription. The screen was designed to test for the presence of a promoter by comparing fluorescence measurements to the FTV native constitutive σ^{70} promoter. The values in this graph are the maximum fluorescent readings after scanning and incubating the samples for 12 hours.



The lower fluorescent readings indicate that those promoters likely interfere with RNA polymerase binding and reduce the transcription rate. Conversely, the "Consensus Promoter 2" appears to have an activating effect upon transcription. In fact, it appears to be activating nearly 2.5 times as much. The remaining promoters yielded almost half as intense fluorescence readings, with minor fluctuations among them.

Discussion

Oxygen sensitive promoters offer an attractive method of controlling genetic transcription. Ambient oxygen content is relatively easy to control and since genetic parts are interchangeable, metabolic engineering applications can take advantage of these promoters. Indeed, controlling protein levels at the transcriptional level is more energetically economical than translational or post-translational control since the cell avoids creating an mRNA strand or polypeptide. As cellular metabolic pathways become understood and optimized, metabolic engineering will increasingly be called upon to produce high quality metabolites for a variety of industries. This field can provide lower operating costs for production facilities by eliminating many of the costly downstream processes. It is therefore critical to develop reliable and predictable genetic parts so researchers and industry can develop a wide array of applications.

References

1. Liao, J.C., Conner, M.R, & Cann, A.F. (2010). 3-methyl-1-butanol production in escherichia coli: random mutagenesis and two-phase fermentation. *Applied Microbiology Biotechnology*, *86*(4), Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2844964/?tool=pubmed
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