Next-Generation Approaches to Overcome the Challenges of Metabolic Pathway Engineering

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Abstract

Engineering metabolic pathways requires answering several questions and often performing many experiments. We sought to answer such "combinatorial questions" using new biophysical models and experimental approaches. First, we employed the CRISPR/Cas9 system to identify the missing enzymes of a metabolic pathway that functions in one organism, but not in another. We demonstrate our approach on a 5-enzyme catabolic pathway that functions in *P. putida*, but not in *E. coli*. Once a multi-enzyme pathway functions in a preferred host, the DNA sequence of the system must then be optimized to achieve a desired metabolic activity. We took a forward engineering approach to systematically investigate the differences in translation rate capacity when using either "frequent", "fast", "rare", or "slow" synonymous codons during codon optimization of protein coding sequences. Our research will enable metabolic engineers to overcome two of the most commonly encountered combinatorial questions during metabolic pathway engineering.

Codon Optimization Objectives

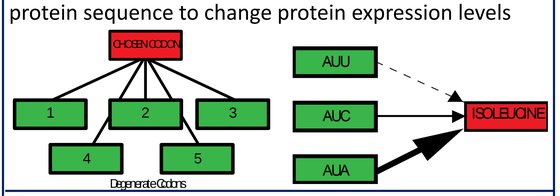
• Search for novel optimization criteria and apply them to a reporter gene

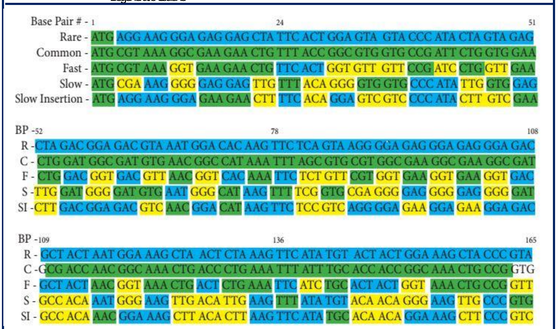
- Express the codon optimized variants and draw conclusions about the effects of non-standard codon optimization methods on translation elongation.
- Investigate an additional point of control for genetic engineering

Optimized superfolder GFP, a gene derived from GFP mut3b by Stephan Milde and Jim Haseloff, iGEM Cambridge 2008

Design

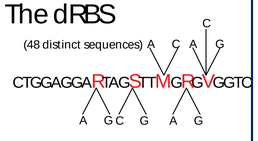
Substitute degenerate codons with higher or lower translation efficiency without altering the amino acid protein sequence to change protein expression levels



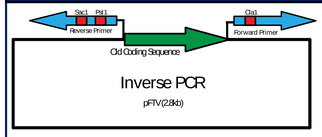


Coding sequence analysis. Blue is for rare, Green is for rare, Yellow is for codons that do not fall under the common or rare category.

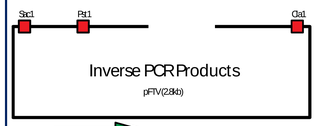
Our degenerate Ribosome Binding Site (dRBS)was designed using the Ribosome Binding Site Calculator (Salis, Nature Biotech 2009). Each GFP coding sequence started with a leader sequence to eliminate discrepancies in translation initiation rate.



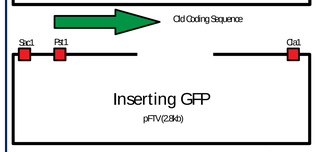
Our Construct

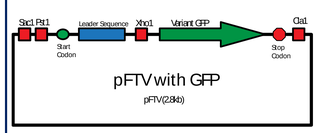


Used pFTV vector to express variant GFPs - contained effective origin of replication, antibiotic resistance marker, promoter, and terminator

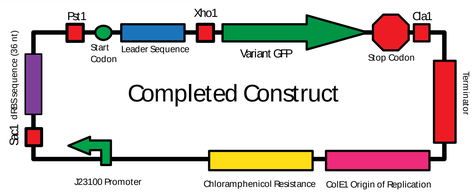


Used inverse PCR to excise the existing GFP, amplify the backbone, introduce new restriction sites to insert a dRBS containing leader sequence and variant coding sequence



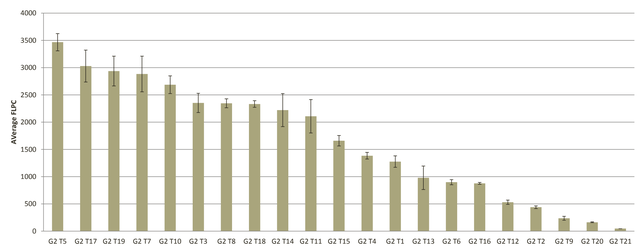


Currently, we have successfully completed all constructs except for slow GFP (4).

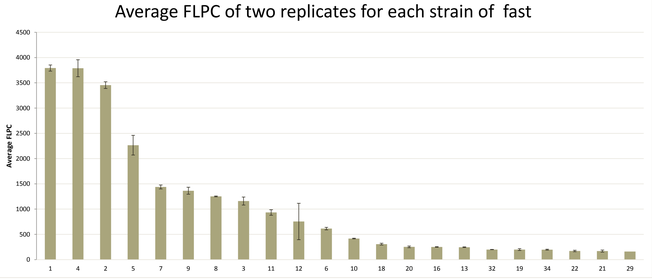


Results

Average FLPC of two replicates for each strain of common



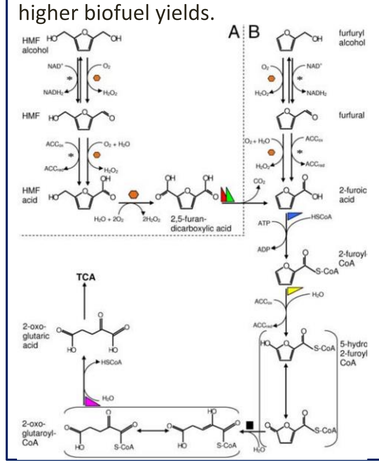
Average FLPC of two replicates for each strain of fast



As of now, common codon optimization results in better expression of superfolder GFP over varying translation initiation rate. Further studies would be to compare and characterize rare and slow codon optimized GFP coding sequence variants. This could lead to more effective ways to repress gene expression for undesirable genes.

Biodetoxification Introduction

Goal: Determine the essential genes of the 5-(hydroxymethyl) furfural (HMF) pathway and engineer *E. coli* with this pathway. Allow for more efficient microbial biofuel production and higher biofuel yields.

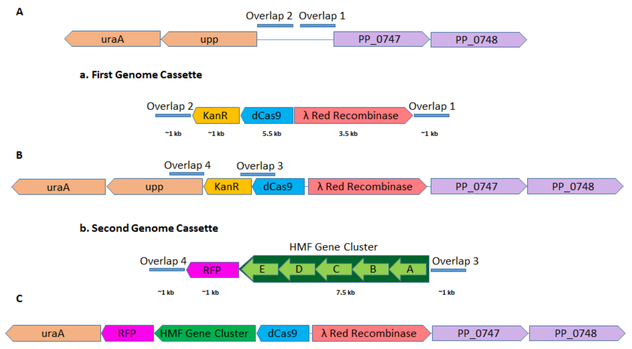


Koopman et. al. identified the 5 gene pathway that detoxifies furan derivatives in *C. basilensis* and *P. putida* – does not function in *E. coli*

HmfABCDE gene cluster breaks down furan derivatives to components of the citric acid cycle

Design and Methods

1) Use Gibson CBA and restriction digests and ligations to assemble plasmid containing dCas9
2) Insert dCas9 (plasmid 1) into *P. putida* genome using homologous recombination

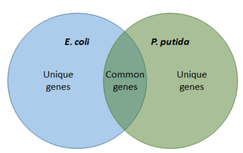


3) Construct plasmid containing HMF pathway, insert into genome
4) Test the function of dCas9 by knocking out RFP and test the function of HMF pathway by doing a furfural consumption assay
5) Assemble CRISPR RNA library and insert into plasmid
6) Conduct gene knockdown experiments using combinatorial dCas9

Combinatorial Gene Knockdown Using dCas9

Iman Farasat identified 19 target genes that could be related to the HMF pathway
Knock out combinations of 2 or 3 genes
Sequence the constructs that no longer consumes furfural

Targets: Cofactors, chaperone proteins, ATPases, accessory proteins

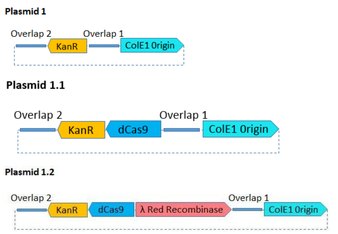


Objectives

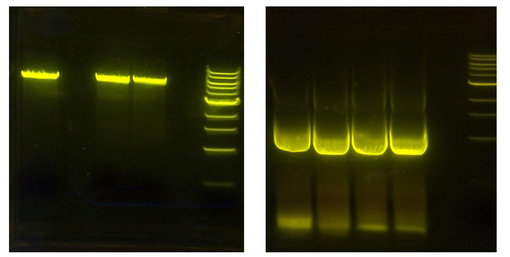
1) Validate the HMF pathway and dCas9 function in *P. putida*
2) Identify the essential genes of the HMF pathway using dCas9 combinatorial gene knockdown
3) Develop a program for automated genome comparisons
4) Engineer the functioning pathway in *E. coli*

Current Progress

Assembled the first plasmid and attempting to insert into the genome via homologous recombination



The kanamycin resistance gene is part of the overlap for the next plasmid containing the HMF pathway
Cannot complete cloning until first plasmid is inserted into genome



dCas9 PCR FTV backbone PCR

Future Steps

Continue attempts to insert HMF and dCas9 into *P. putida* genome
Validate function of dCas9 using RFP knockout
Continue characterization of Slow, Rare GFP variants

Acknowledgements

Dr. Howard Salis and Dr. Tom Richard for helping develop our project ideas
Graduate students Iman Farasat and Chiam Yu Ng for assisting us in wetlab work
Penn State Institutes of Energy and the Environment for funding

References:

(1) Koopman, Frank, et al. "Identification and characterization of the furfural and 5-(hydroxymethyl) furfural degradation pathways of Cupriavidus basilensis HMF14." Proceedings of the National Academy of Sciences 107.11 (2010): 4919-4924.
(2) Graf, Nadja, and Josef Altenbuchner. "Development of a method for markerless gene deletion in Pseudomonas putida." Applied and environmental microbiology 77.15 (2011): 5549-5552
(3) Subramaniam, Arvind R, Tao Pan, and Philippe Cluzel. "Environmental Perturbations Lift the Degeneracy of the Genetic Code to Regulate Protein Levels in Bacteria." Proceedings of the National Academy of Sciences of the United States of America 110.6 (2013): 2419–24. Web. 26 May 2014.