CYANO BRICKS

Expression Testing and Bioproduct Development

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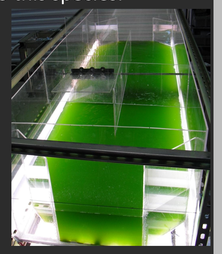
INTRODUCTION

Cyanobacteria are robust platforms for engineering bioproducts. The cyanobacterium Synechocystis sp. PCC 6803 is a model organism in microbiology. But, despite the many potential uses and the promise of PCC 6803 in genetic research, standardized techniques for manipulating and studying bacterial genetics have not been adapted to this species.

Features of Synechocystis:

- Completely sequenced genome
- Capable of natural transformation
- Diverse industrial applications:
  Fuels, plastics, foods, pharmaceutics
- Grows as a photoautotroph or heterotroph
- Carbon neutral manufacturing platform



Photobioreactor

In order to build upon the CyanoBrick tool kit developed by the 2010 Utah State iGEM team, three different high-value bioproducts were targeted for production.

Fatty alcohol, wax ester, and alkane/alkene pathways were developed for integration into PCC 6803. Fatty alcohols and wax esters are used to produce cosmetics, lubricants, and various pharmaceuticals. Alkanes and alkenes are utilized as hydrocarbon fuels. The production of these bioproducts in cyanobacteria will greatly reduce their production cost and increase their availability.

In addition to producing bioproducts, our project also provides a more detailed characterization of the promoters and ribosome binding sites (RBS) in the CyanoBricks toolkit. In order to enhance the usefulness of these promoters, standardized functional testing was done. Utilizing a dual luciferase expression measurement construct, and reference promoters from *E. coli* and Synechocystis, our team more precisely characterized the expression levels of promoters under standard growth conditions. Useful intermediate parts, which are currently not available through the registry, were also produced. This allows the dual luciferase expression measurement system to be easily adapted to new organisms and new reference promoters. A software tool developed by 2010 Utah State iGEM team was used to compare the relative nucleotide similarities between Synechocystis and *E. coli*.

These promoters demonstrated high homology on a nucleotide-by-nucleotide basis for Synechocystis and *E.coli,* which should be indicative of relative functionalities.

```
E. coli sigma70   ---TTGACA----N(15-19)-----TATAAT---

Anderson 1.00     ---TTGACGGCTAGCTCAGTCCTAGGTACAGTGCT
Anderson 0.10     ---TTTATGGCTAGCTCAGTCCTAGGTACAATGCT
Anderson 0.01     ---CTGATGGCTAGCTCAGTCCTAGGGATTATGCT
psbA2             GCTTTACAAAACTCTCATTAATCCTTTAGACTAAG
sigA              CAACTTTGACTGACCAGCTAATTTTGTACACGACT
hspA              CGAATCTAACCTGGAAGGGGAAATTTTAAGATAGA
petBD             CCTTTCCCCAGGGCTTGAAGCTGTGTTACATTTG
```

Promoters Sequence Comparison

```
E. coli  consensus RBS              -----TAAGGAGGT------

Synechocystis consensus RBS         --TTTAAAGGAGGTTAAA--
BBa_B0034 (1.0 Reference RBS)       ---AAAGAGGAGAAA------
psbA2                               AATACTAAGGAATTATAACC
sigA                                ATTATTTGGAGATTTTGCG
hspA                                TCCGTTTAGTTTTTTCAACC
petBD                               GATCCTTAAGAGAAGTCTAG
```

RBS Sequence Comparison

THE SYSTEM

An improved, functional and customizable system for expression measurement was created using a dual luciferase construct, placing the test and control on the same plasmid and in the same organism. This system improves the existing measurement system by including:

- Copy number control
- Growth phase control
- Cell density control

Relative expression levels are calculated using a measured Firefly/*Renilla* (560nm/475nm) ratio, standardized against an Anderson 1.0/Anderson 1.0 reference.

Dual Luciferase Construct

Testing of the system showed expression levels significantly higher than background with little variation among samples taken.

Luminescence in Relative Light Units

Testing Promoter	Firefly RLU (n=3)	*Renilla* RLU (n=3)
Anderson 1.00	45,715,166 ± 19,357,530	642,602,117 ± 150,670,202
Anderson 0.10	3,695,748 ± 769,250	7,889,141 ± 672,672
Anderson 0.01	1,461,220 ± 59,434	916,683 ± 47,130
DH5α (Negative Control)	74 ± 2	174 ± 4

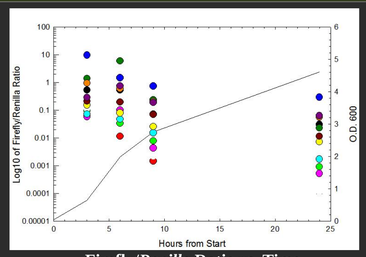
RESULTS

Using only Firefly strengths, the results resemble levels determined by the RFP method. When results were standardized to a 1.0/1.0 reference, promoter strength estimates changed dramatically.

Luminescence Ratios and Resultant Relative Promoter Strengths.

Firefly Promoter (Testing)	*Renilla* Promoter (Reference)	Firefly RLU Average	Firefly Strength	Firefly/*Renilla* Ratio Average	Adjusted Strength
Anderson 1.00	Anderson 1.00	45,715,166	1.000 ± 0.423	0.067	1.00 ± 0.209
Anderson 0.10	Anderson 1.00	3,695,748	0.081 ± 0.017	0.461	6.842 ± 0.901
Anderson 0.01	Anderson 1.00	1,461,220	0.032 ± 0.001	1.673	24.818 ± 1.673
Anderson 0.51/1.00 fusion	Anderson 1.00	5,554,013	0.122 ± 0.047	0.071	1.048 ± 0.071
petBD	Anderson 1.00	162,062	0.004 ± 0.001	0.045	0.661 ± 0.101
sigA	Anderson 1.00	161,684	0.004 ± 0.001	0.235	3.483 ± 1.010
sigA	sigA	143,306	1.000 ± 0.419	0.012	1.000 ± 0.316
hspA	sigA	187,230	1.307 ± 0.387	0.015	1.300 ± 0.269
glnBP2	sigA	331,072	2.310 ± 0.652	0.024	2.031 ± 0.702

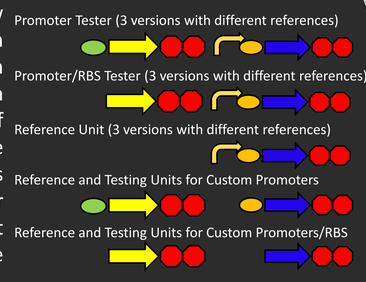
The Firefly/Renilla ratio changed over time as the cell culture aged. This indicates that This may be worthy of further investigation.



Firefly/*Renilla* Ratio vs. Time
Various colors represent different promoters

CUSTOMIZATION

Incorporating the new dual luciferase system into the BioBrick system requires customization capabilities. A suite of intermediate parts are needed to facilitate its utilization in other projects. Promoters that are to be tested are easily interchanged with the existing intermediates along with readily modified ribosome binding sites. Customizable intermediates include a promoter tester, a promoter/RBS tester, a reference unit, reference/testing units for custom promoters and reference/testing units for custom promoters/RBS. Customization allows for testing of promoters in new species.



Dual Luciferase Intermediates

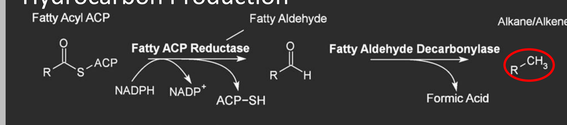
Future developments of this system could include:

- Inverting the *Renilla* reference to assess the effect of terminator read-through.

- Use the Firefly luciferase as reference to verify that results are reporter independent.

BIOPRODUCTS AND PATHWAYS

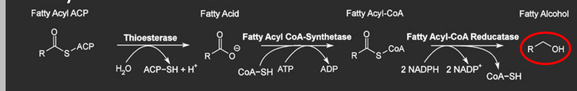
Hydrocarbon Production



Uses of Hydrocarbons:

- Hydrocarbon fuel
- Lubricant components

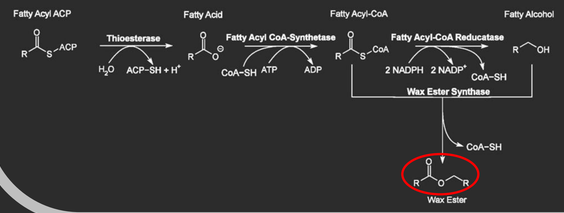
Fatty Alcohol Production



Uses of Fatty Alcohols:

- Precursor to biofuels
- Pharmaceutical applications
- Cosmetic component

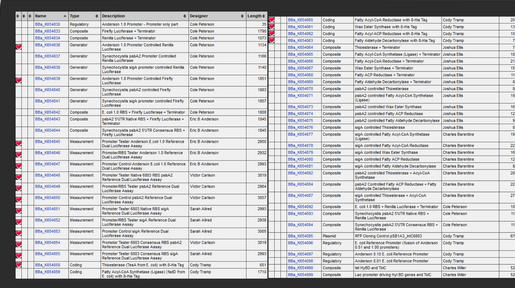
Wax Ester Production



Uses of Wax Esters:

- Waterproofing agent
- Polish Component
- Pharmaceutical applications
- Cosmetic component

CONCLUSIONS



BioBricks constructed and submitted to the Registry

The intent of this project was to: 1. Construct an easily adaptable promoter expression testing method, and 2. Produce useful products in Synechocystis PCC6803. Three pathways involving hydrocarbons, wax esters, and fatty alcohols were developed. Future work will include additional verification and testing of the dual luciferase assay system to establish quantitative promoter performance in addition to relative promoter strength measurements. The system will also be tested in Synechocystis, and a protocol developed for obtaining measurements. Characterization of more promoters in the CyanoBricks toolkit will also allow for a broader range of quantified expression. Product pathways will also be tested using NMR and Mass Spec/G.C. analysis, and finally optimized in Synechocystis.