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Building a Synthetic Biology Toolbox for *Synechococcus* sp. PCC 7002

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Background

Cyanobacteria are excellent hosts for renewable biofuel production, as they naturally fix carbon dioxide to form complex hydrocarbon molecules. The potential for cyanobacterial-based biofuel production has been demonstrated by the genetic engineering of cyanobacteria for the production of a variety of fuels and fuel precursors, including ethanol, butanol, isoprene, free fatty acids (FFAs), and alkanes. While these biofuels have been successfully produced by cyanobacterial hosts, the high titers required for economical production of these low-value commodities have yet to be achieved. Commercial application of cyanobacteria for the production of fuels and other chemicals will require the development of synthetic biology tools for systems-level optimization of cyanobacteria.

Synechococcus sp. PCC 7002 has many characteristics that make this strain ideal for industrial production:

1.*Synechococcus* sp. PCC 7002 has a rapid doubling time (2.6 – 4 h).[1]
2.Large-scale biofuel production systems will be more economical if the algal strain can grow in marine and other non-freshwater sources. Moreover, evaporative losses in algal ponds will further increase the salt concentration. *Synechococcus* sp. PCC 7002 is a marine strain that can tolerate salt concentrations higher than 1.5M (2.5 x seawater).[2]
3.Algal ponds and PBRs in biofuel production systems will also be exposed to high light intensities during peak sunlight hours. *Synechococcus* sp. PCC 7002 displays extreme high light tolerance, surviving under light intensities as high as 2 x peak sunlight (4.5 mE m^{-2} s^{-1}).[3]
4.Lastly, ponds and PBRs are also exposed to large temperature swings, with high temperatures in PBRs being particularly problematic. *Synechococcus* sp. PCC 7002 has a wide temperature range of growth (22-40°C).[2]

Objectives

Goal: To develop synthetic biology tools to enable systems-level optimization of *Synechococcus* sp. PCC 7002 for industrial applications.

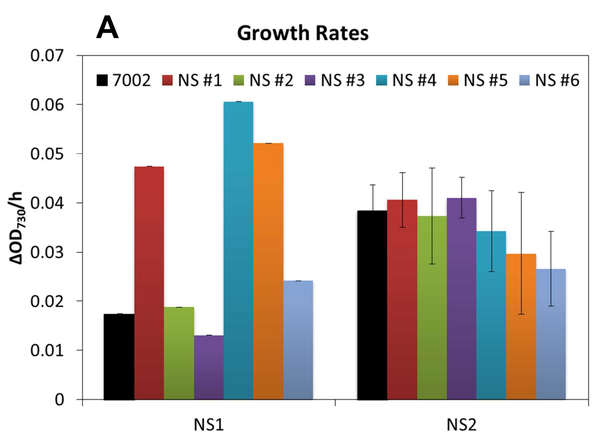
- Identify 'neutral' integration sites for genome integration of target genes
- Optimize the transformation process for efficient DNA integration and mutant isolation
- Characterize reporter proteins for quantification of gene expression
- Develop gene expression tools for transcriptional optimization

Results

Identification of 'Neutral' Integration Sites

Table 1: *Synechococcus* sp. PCC 7002 genome locations with no predicted function.

Neutral Site	Start	End	Size (bp)	Flanking Genes
NS1	963217	964242	1026	DnaJ domain protein; hypothetical protein
NS2	1247018	1248056	1039	*sigB,* sigma-70 factor; aspartate aminotransferase
NS3	1864422	1265821	1400	*cas2*, CRISPR-associated protein; tRNA-Met



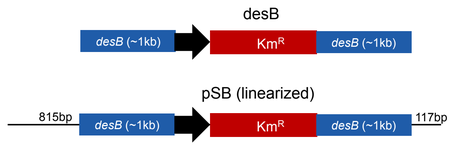
Figures 1A-C: Linear growth rates (A), final cell concentrations (B), and final photosynthetic yields (C) for *Synechococcus* sp. PCC 7002 wild type, NS1 mutants, and NS2 mutants. For each NS target, six different transformants were analyzed. NS1 mutant is spectinomycin resistant; NS2 mutant is kanamycin resistant. Growth temperature = 34°C, illumination = 60 µmol photons m^{-2} s^{-1}, agitation = 150 rpm. NS1 data is one biological replicate; NS2 data is an average of 3 biological replicates with error bars indicating standard deviation.

- NS1 and NS2 mutants do not show significant changes in growth rate, final cell concentration, and photosynthetic yield compared to the wild type (7002).

Results (cont.)

Transformation Optimization

Exonuclease Activity



- Transformation efficiency = # transformed cells / µg DNA
- A more than 30-fold improvement in transformation efficiency with linear pSB indicates that exonucleases are active in *Synechococcus* sp. PCC 7002

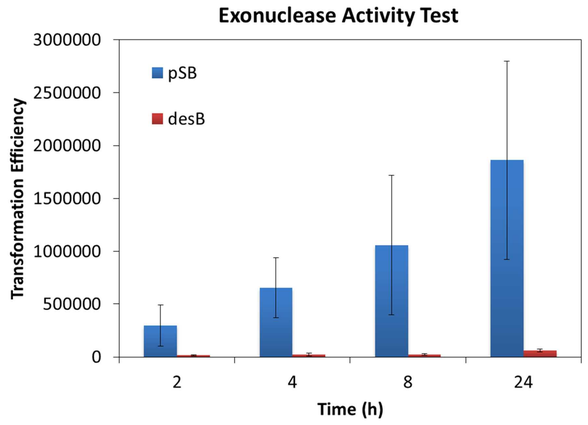


Figure 2: Transformation efficiency of *Synechococcus* sp. PCC 7002 using linear DNA fragments with (pSB) and without (desB) protection nucleotides to test for exonuclease activity. Cell concentration (OD730 = 1.0), DNA = 0.5 µg. Data are averages of 3 biological replicates.

Transformation Conditions

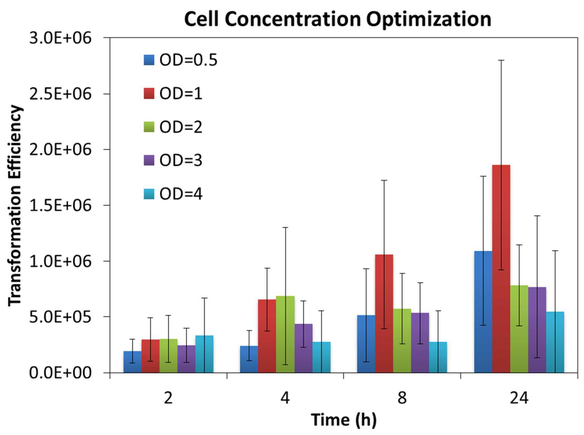


Figure 3: Transformation efficiency of *Synechococcus* sp. PCC 7002 with varying cell concentration. DNA fragment = 0.5 µg of linearized pSB. Data are averages of 3 biological replicates.

- OD_{730} = 1.0 is an optimal cell concentration for high transformation efficiency
- Other environmental conditions will be investigated using design of experiments:
  - DNA concentration
  - Growth phase
  - Light intensity
  - Temperature
  - Agitation
  - Length of homologous regions
- Conditions must also minimize the number of genomes per cell

Fluorescent Protein Reporters and Gene Expression Tools

- Native absorbance and fluorescence of *Synechococcus* sp. PCC 7002 must be avoided when selecting a fluorescent protein reporter. (Figure 4)
- 3 fluorescent proteins were selected for codon optimization and DNA synthesis.
- Native *Synechococcus* sp. PCC 7002 promoters were selected based on RNA-seq results for: varying expression levels as well as constitutive, exponential phase, and stationary phase expression. (Figure 5)

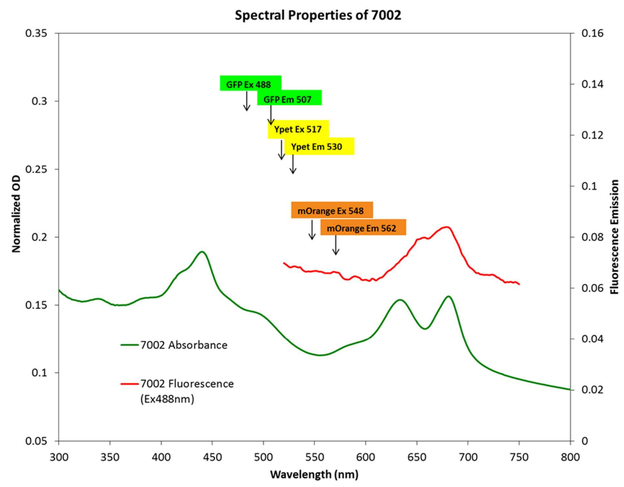
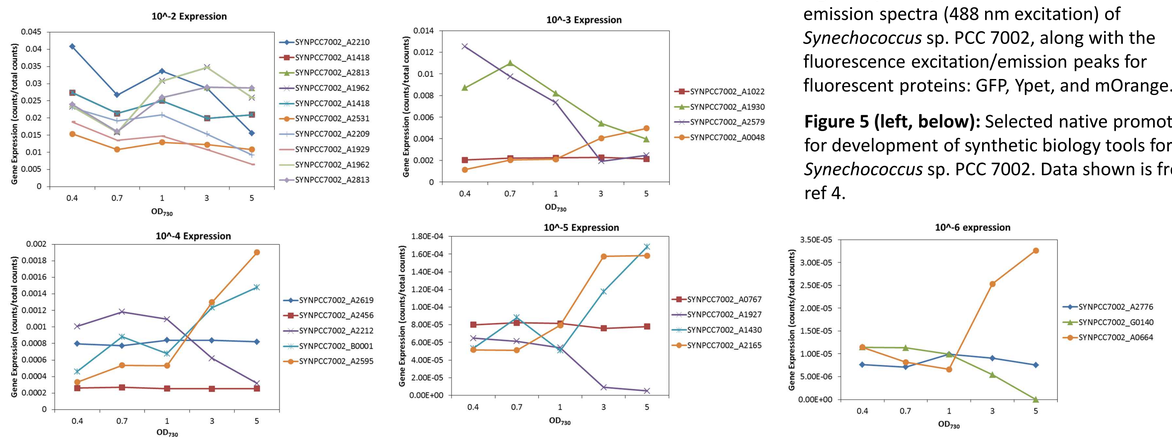


Figure 4 (above): Absorbance and fluorescence emission spectra (488 nm excitation) of *Synechococcus* sp. PCC 7002, along with the fluorescence excitation/emission peaks for fluorescent proteins: GFP, Ypet, and mOrange.

Figure 5 (left, below): Selected native promoters for development of synthetic biology tools for *Synechococcus* sp. PCC 7002. Data shown is from ref 4.



Conclusions

- Identified and confirmed 2 neutral integration sites; testing of third neutral site is underway.
- Determined exonuclease activity is present in *Synechococcus* sp. PCC 7002.
- OD_{730} = 1.0 is an optimum concentration for transformation of *Synechococcus* sp. PCC 7002.
- Identified reporters and promoters as tools for engineering *Synechococcus* sp. PCC 7002.

Reference & Acknowledgements

1. Bryant DA. *Synechococcus sp.* PCC 7002: a Robust, Versatile, and Cosmopolitan Cyanobacterial Platform for Biofuels Production. http://bioenergy.asu.edu/cb&p/abstracts/Bryant-abstract.html.
2. Ludwig M, Bryant DA. 2012. *Synechococcus* sp. strain PCC 7002 transcriptome: Acclimation to temperature, salinity, oxidative stress, and mixotrophic growth conditions. Frontiers in Microbiol. 3:354.
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4. Ludwig M, Bryant DA. 2011. Transcription profiling of the model cyanobacterium *Synechococcus* sp. strain PCC 7002 by Next-Gen (SOLiD™) sequencing of cDNA. Frontiers in Microbiol. 2:41.

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