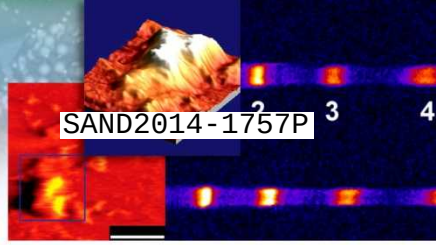
SANDIA BIOSCIENCE



Synechococcus sp. PCC 7002: A Cyanobacterial Chassis for Advanced Biofuel Production

Anne M. Ruffing (8622)
Senior Member of the Technical Staff

March 5, 2014

BSAP Annual Review

Goal of Sandia's Bioscience Research Foundation

We analyze, understand, and control the functions of biological systems to meet National Security challenges in biodefense and biofuels production.

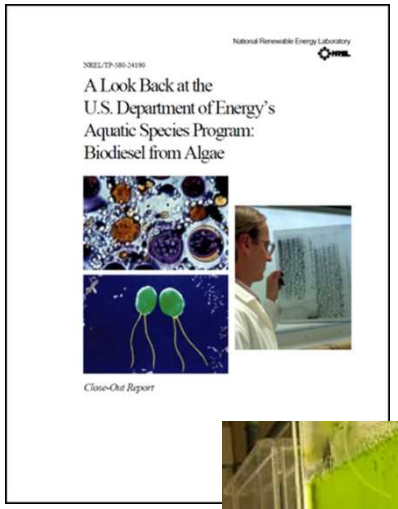
- Analyze: Analyze samples to obtain the most relevant biological data for biodefense and biofuels.
- Understand: Integrate data sets and use them to build predictive models of biological systems.
  - Decipher molecular mechanisms relevant to biofuels or biodefense.
  - Recognize biological correlations and dependencies to identify unique signatures, markers and/or design new pathways.
- Control: Rationally modify the functions of biological systems.
  - Bioenergy: Demonstrate and optimize efficient, cost-effective production of drop-in fuels and co-products from the conversion of lignocellulose or algae.
  - BEID: Design and deploy countermeasures and materials which are safer and/or more effective than traditional solutions

SANDIA BIOSCIENCE

Cyanobacterial-Based Biofuel Production

Desirable strain traits

- Easily transformed
- Homologous recombination – targeted genome integration
- Gene expression not complicated by RNAi
- Established genetic tools
- Fast growth rates and strain robustness



Process design advantages

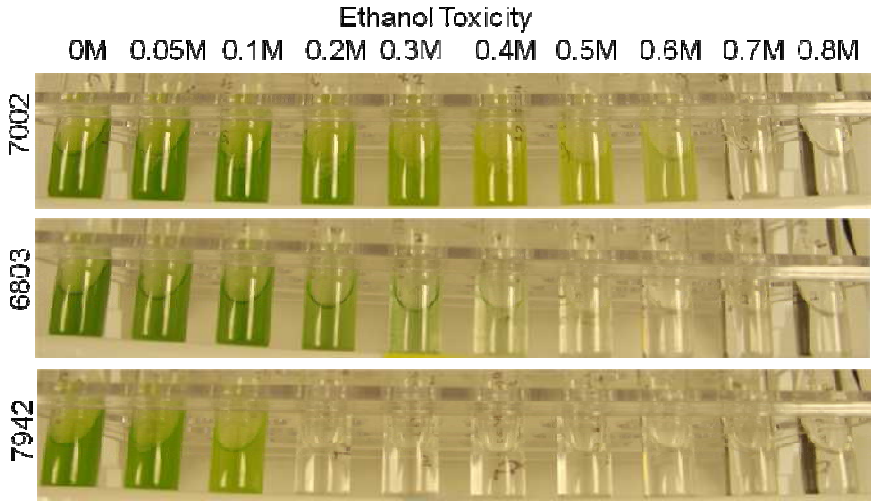
- Product excretion enables continuous production
- Biomass harvesting not required
- Lower nutrient requirements (N&P)

Cyanobacterial-based biofuel production has numerous advantages compared to traditional algal biofuels.

SANDIA BIOSCIENCE

Synechococcus sp. PCC 7002

- Model cyanobacterium
  - Genetic tools available
  - Genome sequence
- Rapid doubling time (2.6 – 4 hrs)
- Temperature tolerance
  - Grows under 22-40°C
- Salt tolerance
  - Survives > 1.5 M NaCl (2.5x sea water)
- High light tolerance
  - Survives light intensities > 4.5 mE m^{-2} s^{-1} (2x peak sunlight)
- Biofuel tolerance
  - Grows in ethanol concentrations up to 0.6 M



Synechococcus sp. PCC 7002 has many desirable traits for advanced biofuel production.

SANDIA BIOSCIENCE

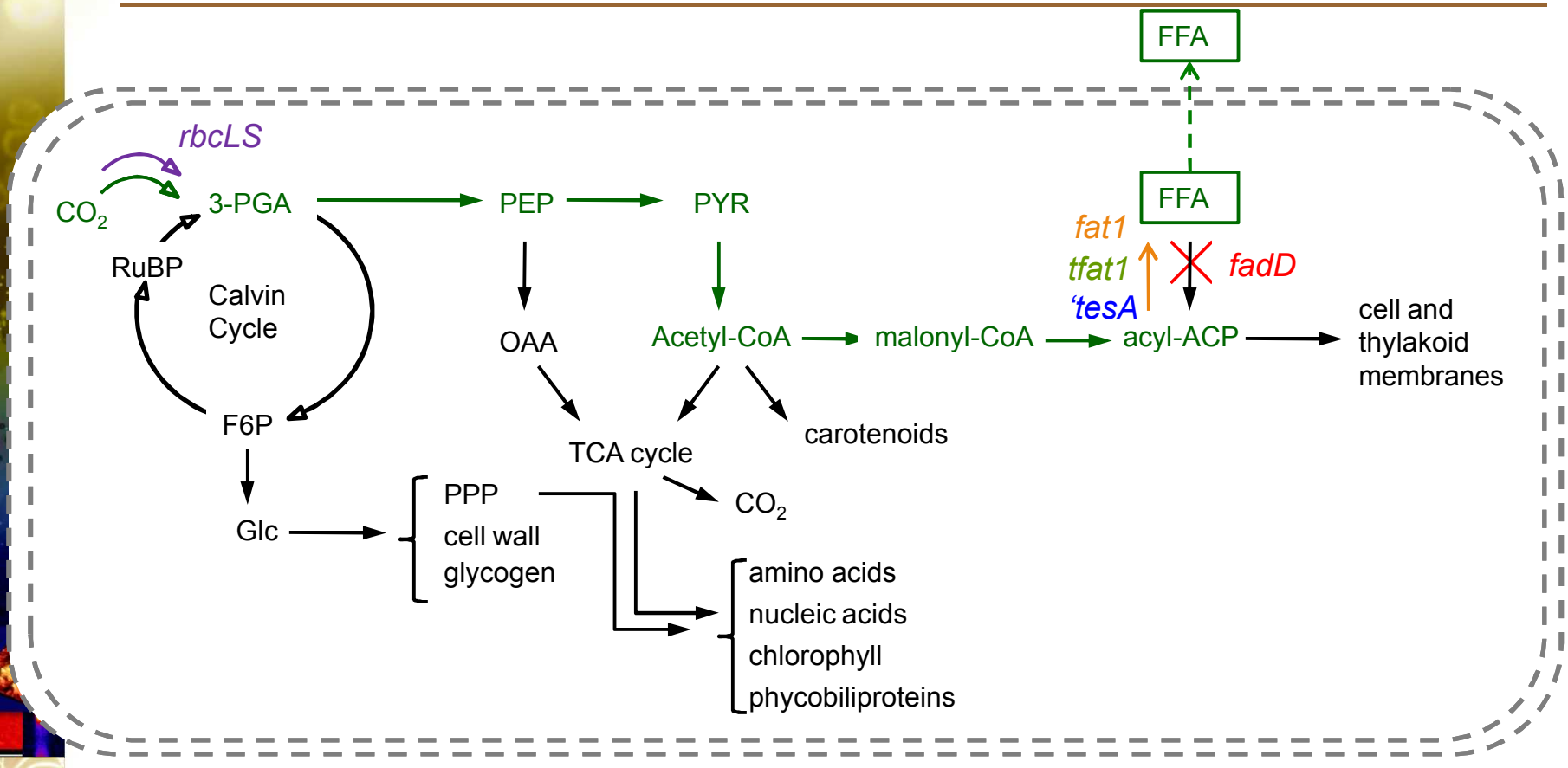
Sandia National Laboratories

Engineering *Synechococcus* sp. PCC 7002 for Advanced Biofuel Production

- Traditional Metabolic Engineering of *Synechococcus* sp. PCC 7002 for Free Fatty Acid Production (Funding: Truman Fellowship)
- Systems-Level Metabolic Engineering of *Synechococcus* sp. PCC 7002 for Alkane Production (Funding: Early Career LDRD)

SANDIA BIOSCIENCE

Traditional Metabolic Engineering of *Synechococcus* sp. PCC 7002 for FFA Production

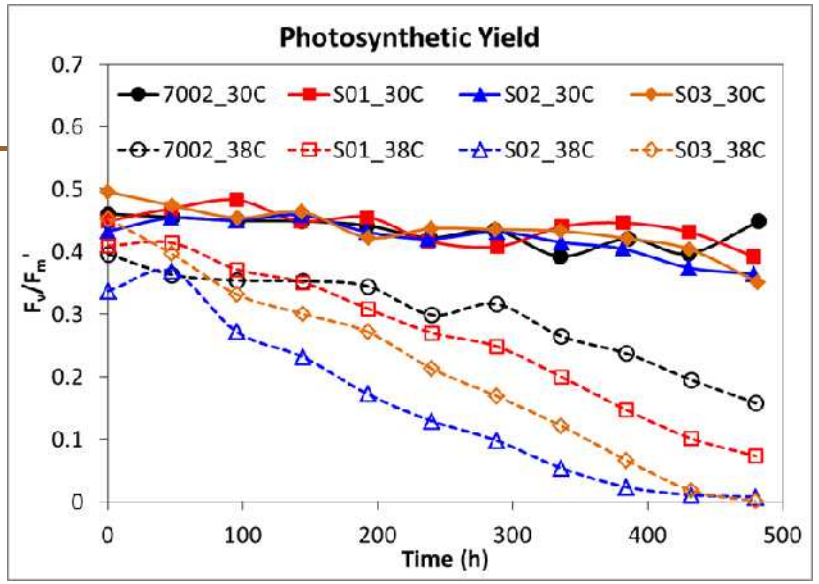


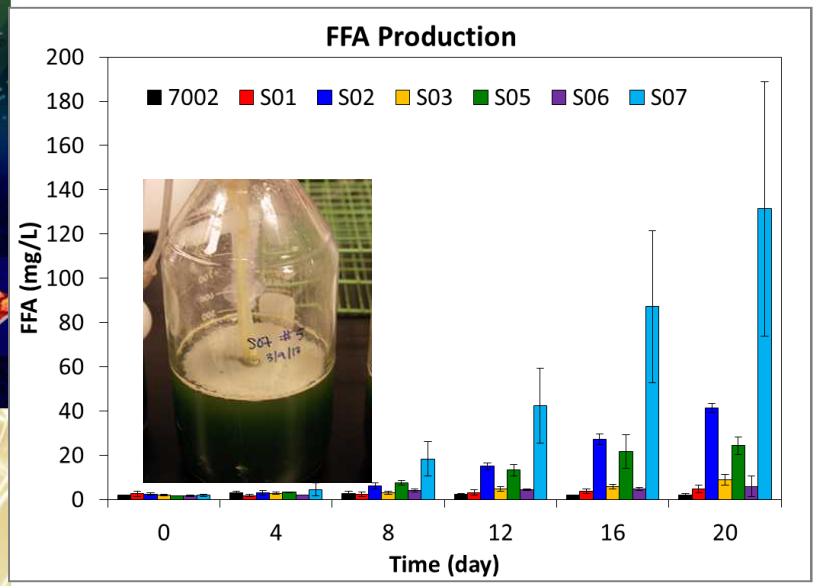
7002: wild type; S01: Δ*fadD*; S02: Δ*fadD, 'tesA*; S03: Δ*fadD, fat1*; S05: Δ*fadD, tfat1*; S06: Δ*fadD,* Ptrc-*'tesA*, *rbcLS*; S07: Δ*fadD, 'tesA*, PpsbA1-*rbcLS*

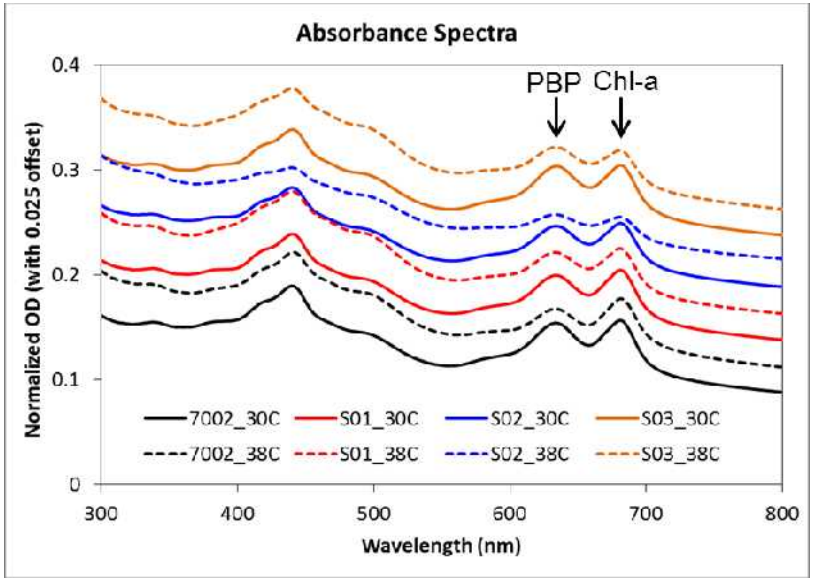
Six engineered strains of Synechococcus sp. PCC 7002 were constructed for FFA production.

FFA Production

- Maintenance of high photosynthetic yields and photosynthetic pigments (PBP, Chl-a) is temperature dependent
- Significant increase in FFA production with overexpression of RuBisCO from *psbAI* promoter







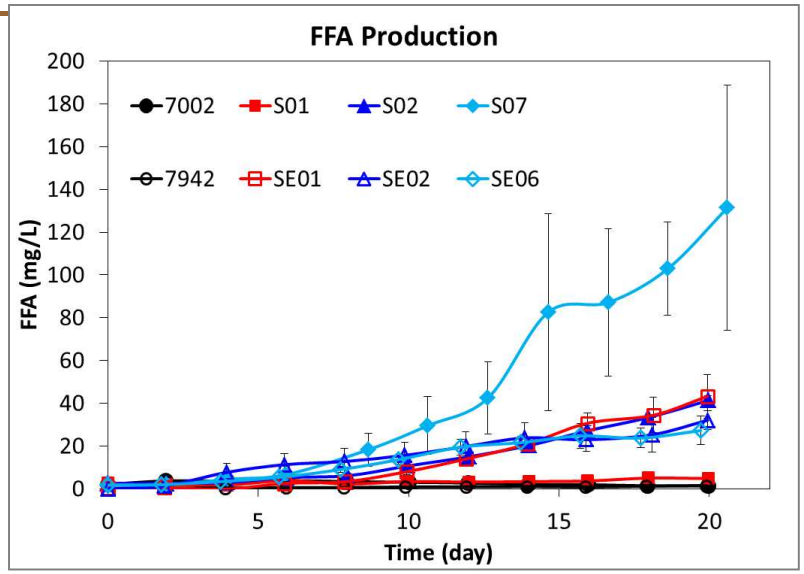
High temperatures and high FFA production had negative impacts on the physiology of the host, Synechococcus sp. PCC 7002.

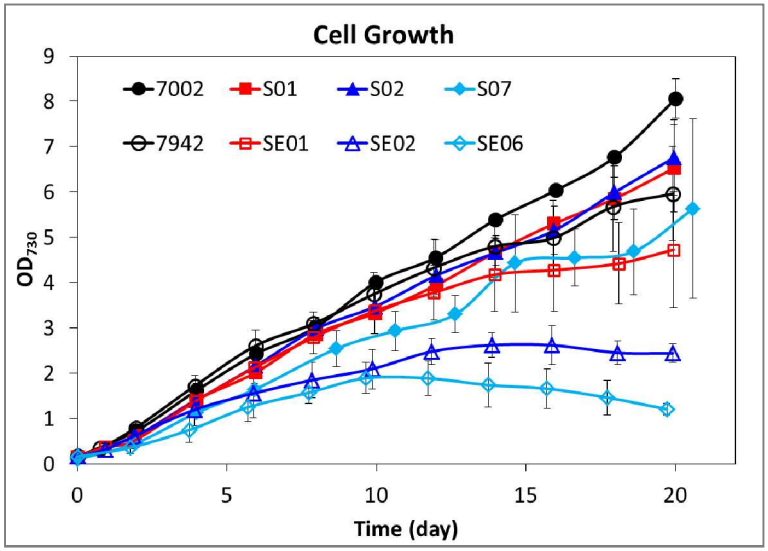
SANDIA BIOSCIENCE

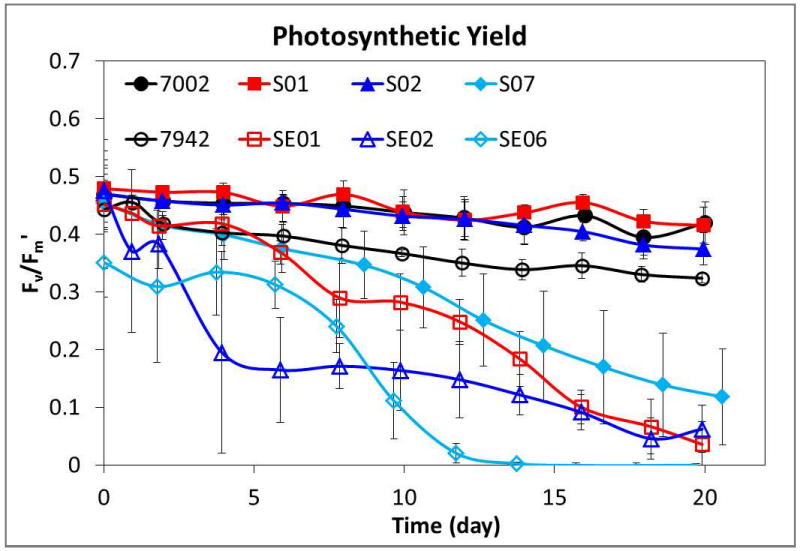
Sandia National Laboratories

Host Selection: *Synechococcus* sp. PCC 7002 vs *Synechococcus elongatus* PCC 7942

- Highest FFA producing strains:
  - 7002: S07 (131 mg/L)
  - 7942: SE01 (43 mg/L)
- Improved growth and photosynthetic yields for 7002 strains





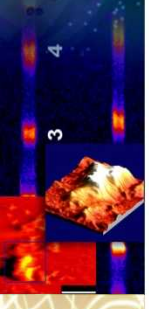


Synechococcus sp. PCC 7002 is an advantageous host for FFA production.

SANDIA BIOSCIENCE

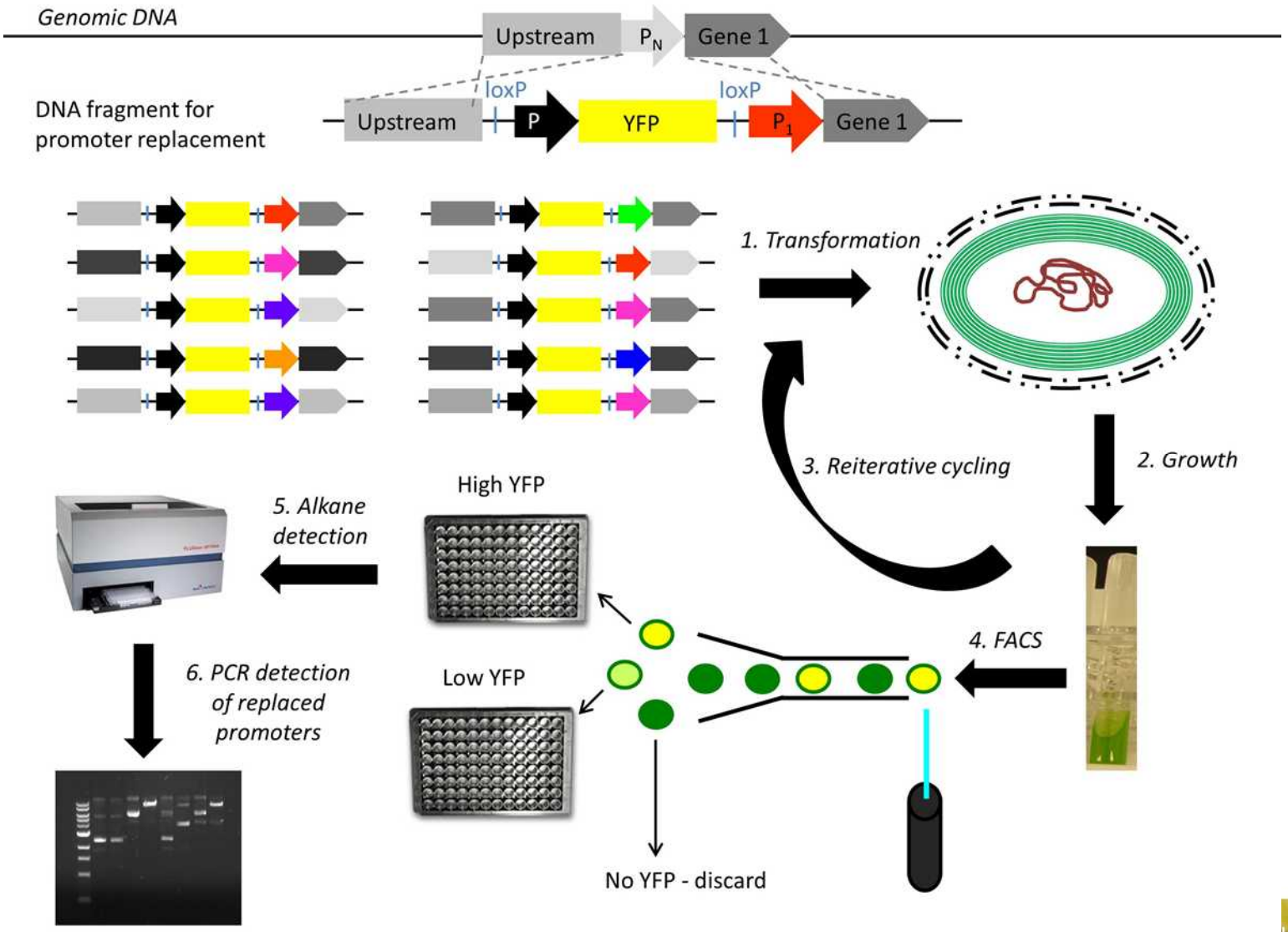
Traditional Metabolic Engineering: Conclusions

- *Synechococcus* sp. PCC 7002 is a better host for FFA production compared to *S. elongatus* PCC 7942
- Carbon fixation (RuBisCO) limits FFA biosynthesis in *Synechococcus* sp. PCC 7002
- Gene expression is critical for optimizing FFA production
- FFA production has complex, systems-level interactions (growth, photosynthetic yield, pigments) that cannot be optimized by traditional pathway engineering



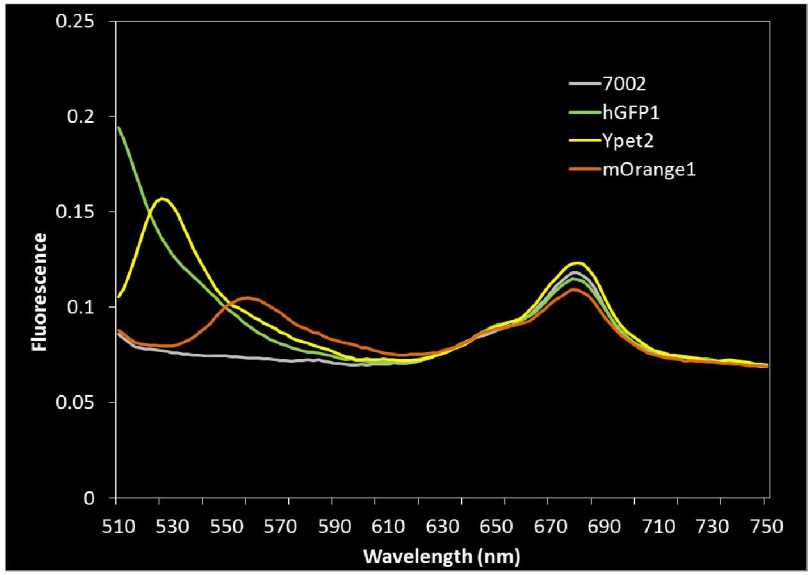
SANDIA BIOSCIENCE

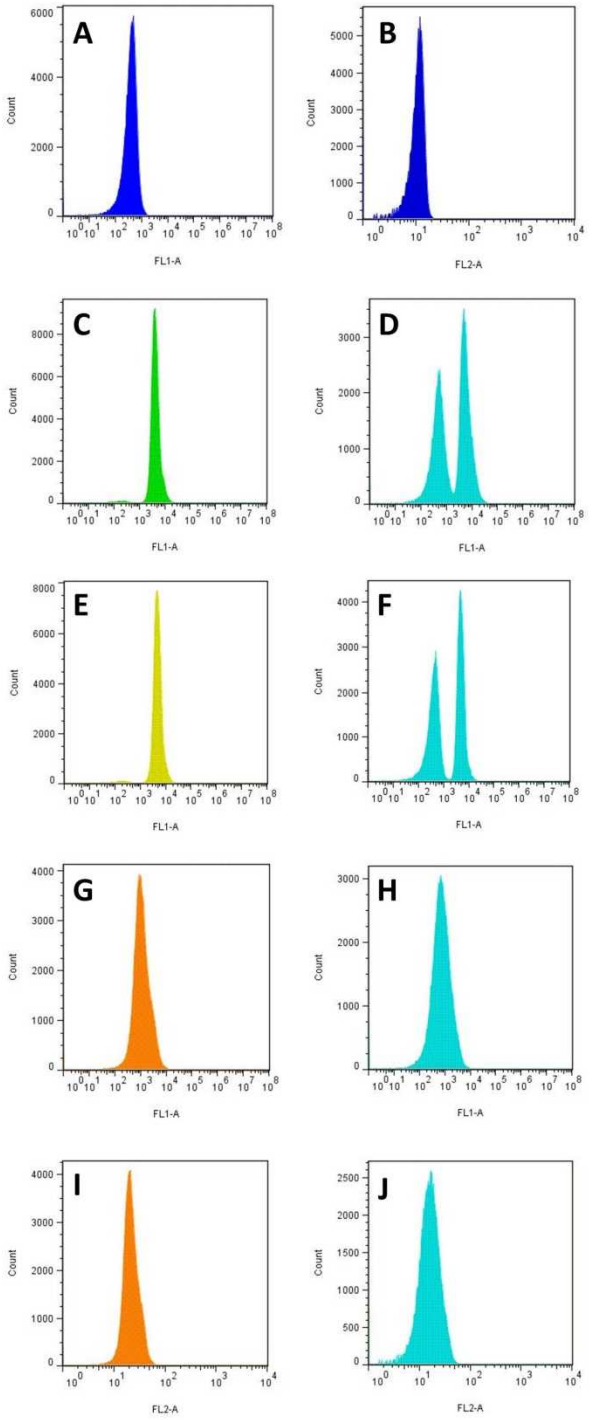
Development of a Systems-Level Metabolic Engineering Technique for *Synechococcus* sp. PCC 7002



Fluorescent Protein Reporter Selection

- 3 fluorescent proteins investigated: GFP, YFP, and OFP
- YFP has the best combination of :
  - high signal intensity
  - minimal overlap with cyanobacterial photosynthetic pigments
  - good flow cytometer peak separation (488nm excitation laser)
  - excitation (514nm) and emission (527nm) peaks



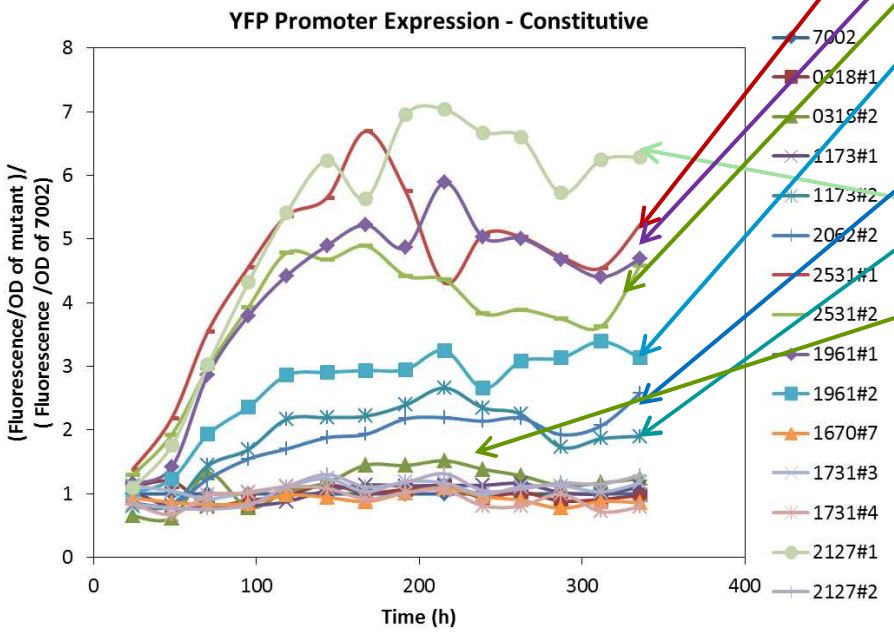


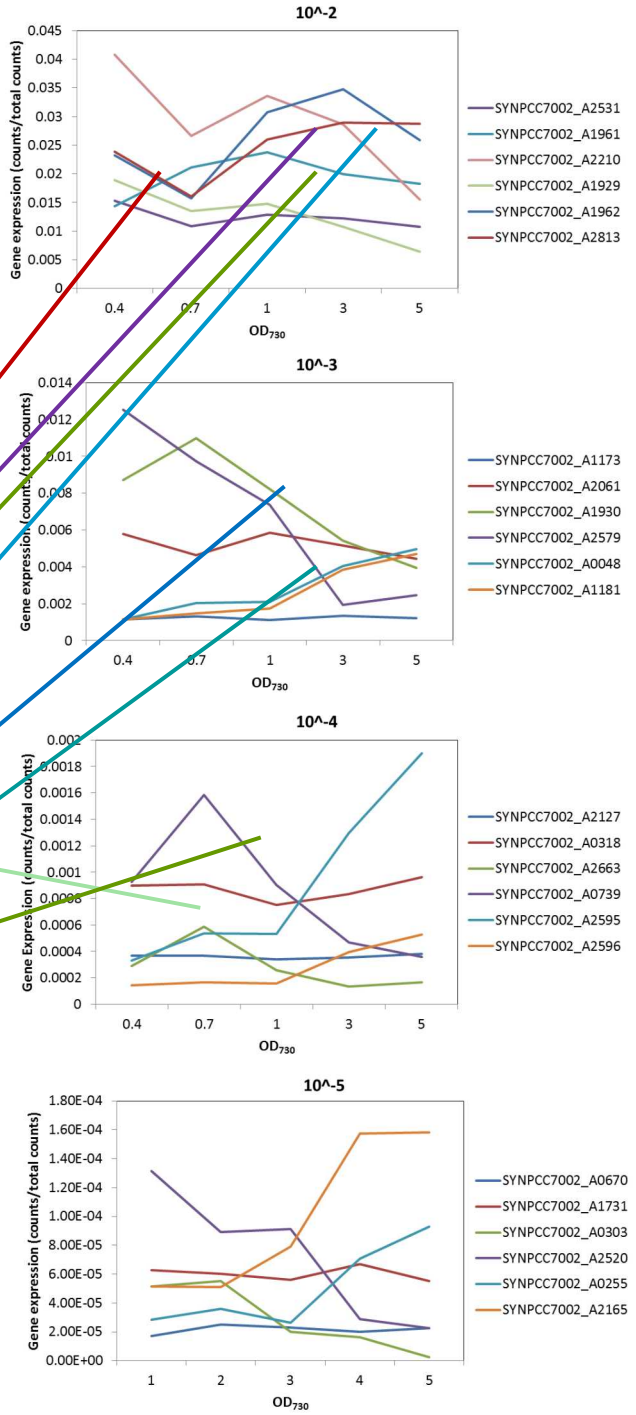
YFP is a good reporter in Synechococcus sp. PCC 7002.

SANDIA BIOSCIENCE

Promoter Characterization in *Synechococcus* sp. PCC 7002

- Promoters selected from previously published RNAseq data (Ludwig, Bryant. 2011. Frontiers in Microbiol. 2:41.)
  - Expression levels: 10^{-5} to 10^{-2}
  - Regulatory patterns: constitutive, linear growth phase, and stationary phase
- Linked promoters to YFP for characterization under continuous light and diurnal conditions



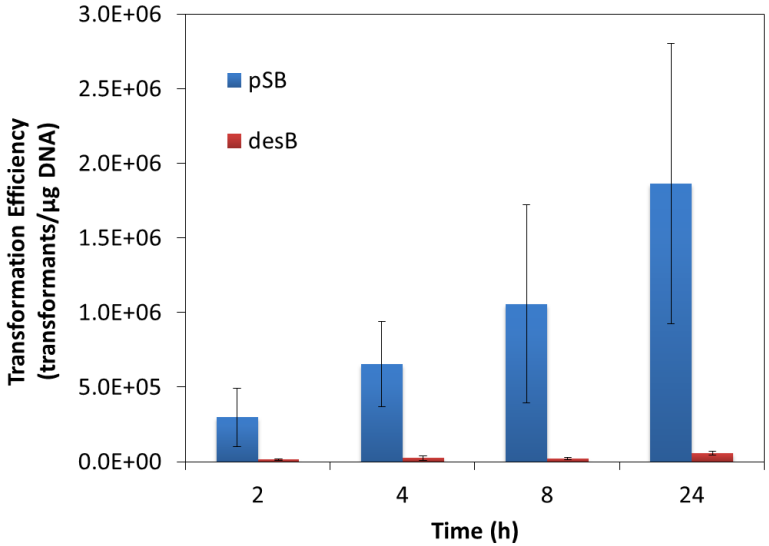


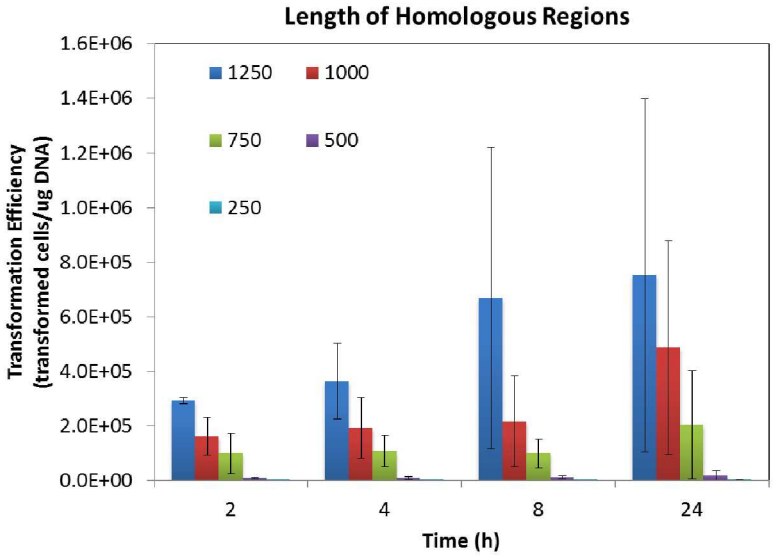
Constitutive promoters were identified, cloned, and characterized in Synechococcus sp. PCC 7002.

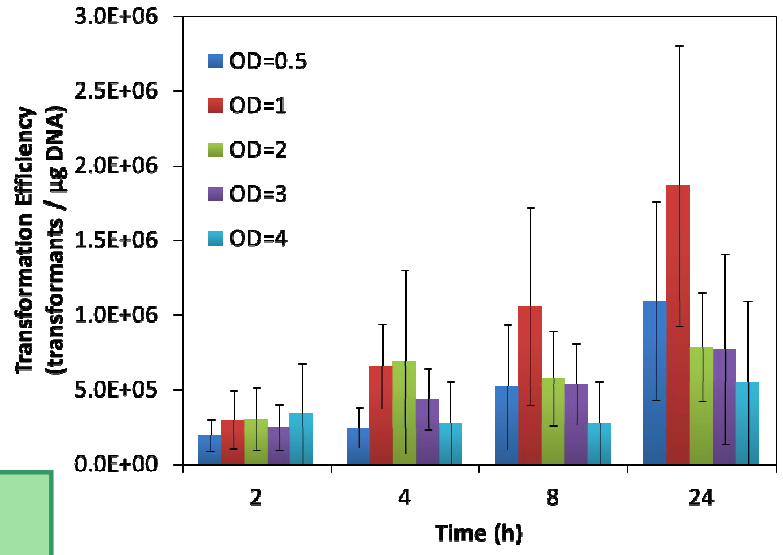
SANDIA BIOSCIENCE

Optimizing Transformation in *Synechococcus* sp. PCC 7002

- Exonuclease activity present in *Synechococcus* sp. PCC 7002
- Cell concentration: OD_{730} = 1.0
- Length of homology for homologous recombination: 1kb
- Genome copy number (ploidy): lowest at 34°C, 60 μmol*m^{-2}*s^{-1}, OD_{730} > 3.0







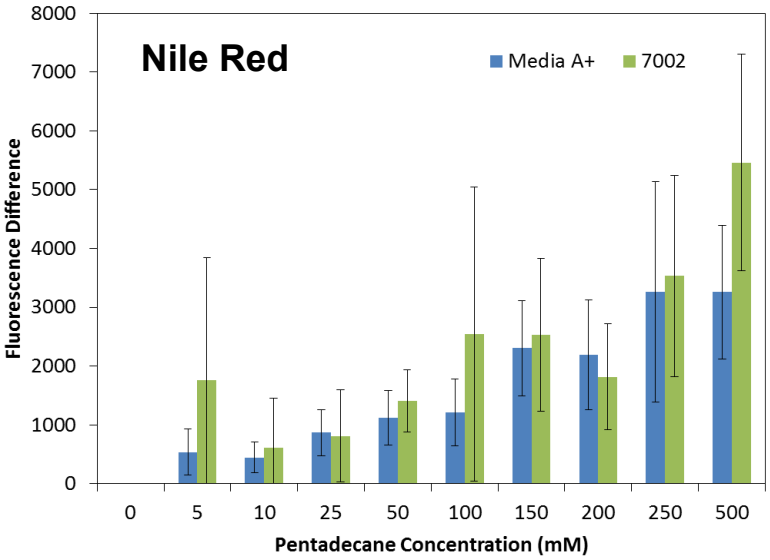
Factors for high transformation efficiency in Synechococcus sp. PCC 7002 were optimized.

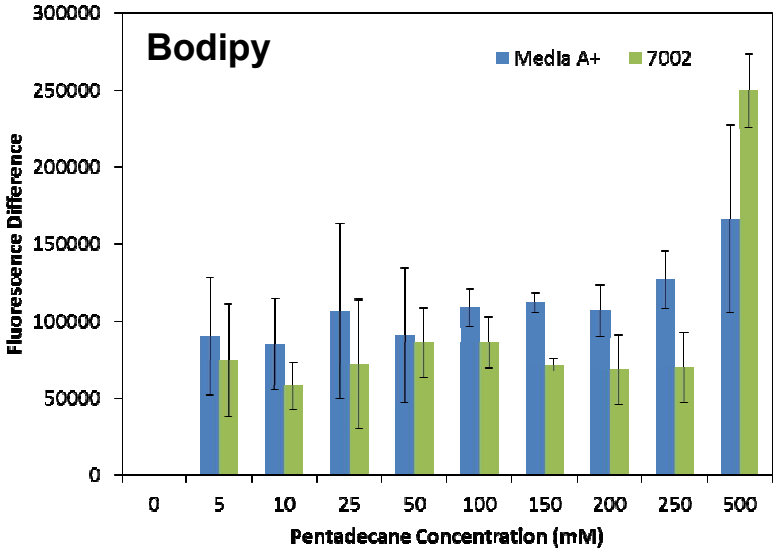
SANDIA BIOSCIENCE

Sandia National Laboratories

High-Throughput Alkane Screening

- 2 neutral lipid dyes: nile red and bodipy
- Exogenous addition of pentadecane to media or dilute culture (OD_{730} = 0.1)
- Nile red is more quantitative
- Bodipy is more sensitive





Neutral lipid dyes can be used for high-throughput screening of alkane production.

SANDIA BIOSCIENCE

Sandia National Laboratories

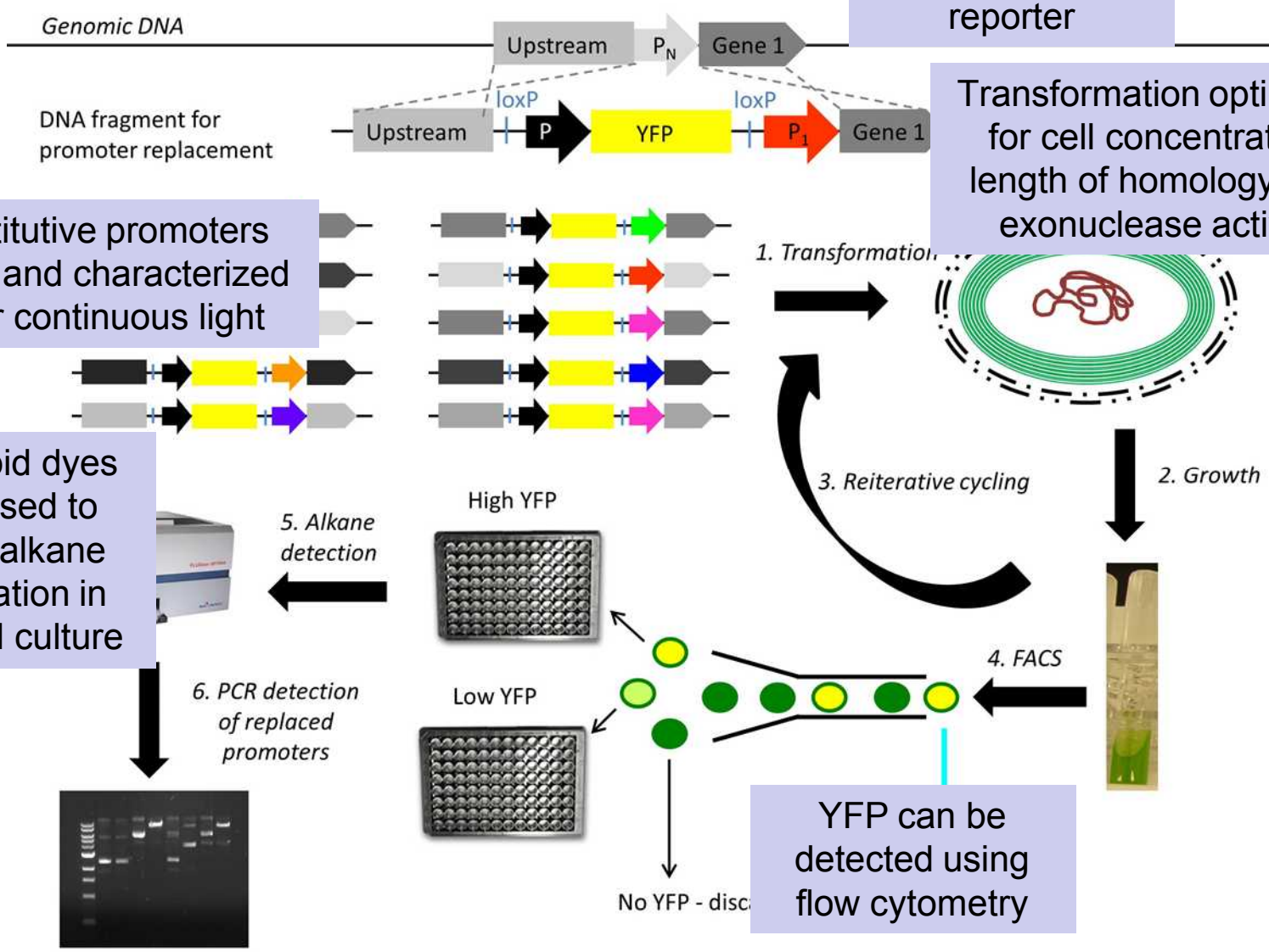
Systems-Level Metabolic Engineering: Summary

YFP selected as reporter

Transformation optimized for cell concentration, length of homology, and exonuclease activity

Constitutive promoters cloned and characterized under continuous light

Neutral lipid dyes can be used to estimate alkane concentration in media and culture



YFP can be detected using flow cytometry

SANDIA BIOSCIENCE

Laboratories

Systems-Level Metabolic Engineering: Future Work

- Clone and characterize linear growth phase and stationary phase expression promoters
- Determine conditions for minimal genome copy number
- Analyze fluorescence detection of multiple YFP copies
- Characterize neutral lipid stains for intracellular alkane production
- Design synthetic DNA constructs for systems-level metabolic engineering (JGI DNA synthesis proposal pending)
- Demonstrate systems-level metabolic engineering for improving alkane production in *Synechococcus* sp. PCC 7002

SANDIA BIOSCIENCE

Publications

Anne M Ruffing and Christine A Trahan. Biofuel Toxicity and Mechanisms of Biofuel Tolerance in Three Model Cyanobacterial Strains. To be submitted to *Algal Research* (June 2014).

Anne M Ruffing. Improved Free Fatty Acid Production in Cyanobacteria with *Synechococcus* sp. PCC 7002 as Host. To be submitted to *Frontiers in Bioengineering and Biotechnology* (March 2014) – Research Topic: Cyanobacteria, the green *E. coli.*

Anne M Ruffing. RNA-Seq analysis and targeted mutagenesis for improved free fatty acid production in an engineered cyanobacterium. *Biotechnology for Biofuels*. 2013. 6:113.

Anne M Ruffing. --Borrowing genes from *Chlamydomonas reinhardtii* for free fatty acid production in engineered cyanobacteria. *Journal of Applied Phycology.* 2013. 25(5): 1495-1507.

Anne M Ruffing. Metabolic Engineering of Hydrocarbon Biosynthesis for Biofuel Production. Book chapter in *Liquid, Gaseous and Solid Biofuels – Conversion Techniques*. Intech, Rijeka, Croatia. 2013.

Anne M Ruffing and Howland DT Jones. Physiological effects of free fatty acid production in genetically engineered *Synechococcus elongatus* PCC 7942. *Biotechnology and Bioengineering*. 2012. 100(9): 2190-2199. (Cover, Spotlight)

Anne M Ruffing. Engineered Cyanobacteria: Teaching an old bug new tricks. *Bioengineered Bugs*. 2011. 2(3).

Acknowledgements

Staff Members

Howland D.T. Jones (entrepreneurial leave)

Omar F. Garcia (now in 5448)

Student Interns

Christine Trahan

Travis Jensen

Michelle Raymer

Managers

Anthony Martino (now in 6124)

Eric Ackerman (8635)

James Carney (8622)

Funding

President Harry S. Truman Fellowship in National Security Science and Engineering (LDRD): 01/25/2010 – 01/24/2013

Early Career LDRD: 04/01/2013 - present

SANDIA BIOSCIENCE