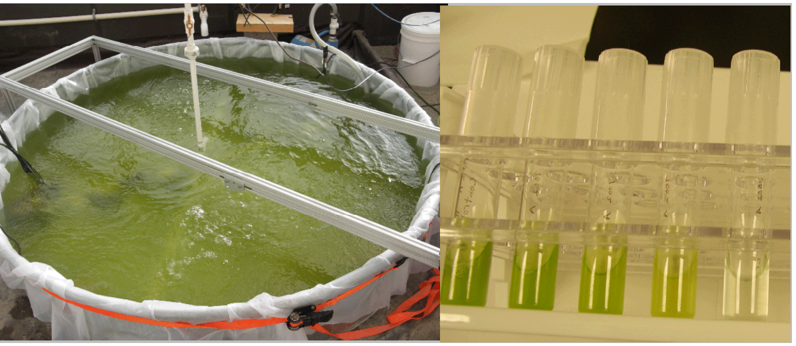
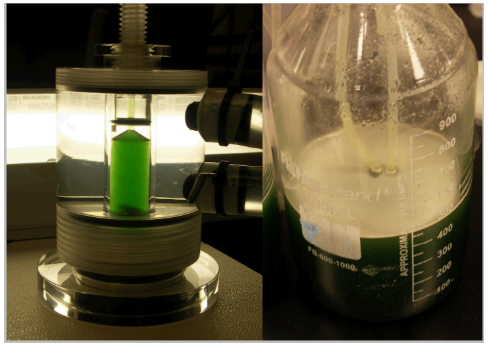
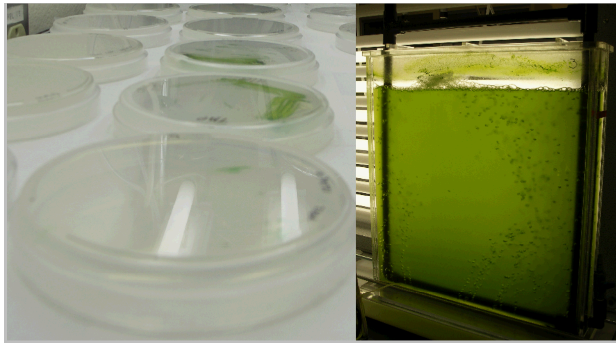
Exceptional service in the national interest

Sandia National Laboratories







Cyanobacterial Biofuels: The Blue-Green Revolution

Anne Ruffing

April 28, 2015

U.S. DEPARTMENT OF ENERGY

NNSA National Nuclear Security Administration

Sandia National Laboratories is a multi-program laboratory managed and operated by Sandia Corporation, a wholly owned subsidiary of Lockheed Martin Corporation, for the U.S. Department of Energy's National Nuclear Security Administration under contract DE-AC04-94AL85000. SAND NO. 2011-XXXXP

President Harry S. Truman Fellowship in National Security Science and Engineering

- Three-year Truman Fellowship allows creativity and stimulates exploration of forefront science and technology and high-risk, potentially high-value R&D in research areas of importance to Sandia—at either Livermore, CA or Albuquerque, NM sites
- Candidate requirements:
  - Solved a major scientific or engineering problem or provided a new approach to a major problem, as evidenced by a recognized impact in their field
  - Must be within 3 years of receiving their PhD and be seeking their first national laboratory appointment (pre postdoc internship excluded)
  - 3.5 undergraduate and 3.7 graduate GPA preferred
  - Must be a US citizen
- $111,200 starting salary, plus benefits and additional funding for research
- Application deadline: November 1, yearly
- Selection in March for Fall start
- http://www.sandia.gov/careers/fellowships.html#truman

Energy Security

- Energy impacts U.S. economy
- Department of Defense relies on petroleum for approximately 77% of its energy needs[1]
- Threats to energy security:
  - Political instability of oil producing countries
  - Manipulation or attacks on energy supply
    - 1973 Arab oil embargo
    - Algerian oil field attacked (2013)
    - ISIS attack on Iraq oil refinery (2015)
  - Accidents or natural disasters
    - BP Deepwater Horizon oil spill (2010)
    - Hurricane Katrina (2005)
  - Increasing demand, finite supply

1. Parthemore C. (2010). *Fueling the Force: Preparing the Department of Defense for a Post-Petroleum Era*. Center for New American Security.

Sandia National Laboratories

National Renewable Energy Laboratory

NREL/TP-580-24190

A Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae

Cyanobacteria. This group is prokaryotic, and therefore very different from all other groups of microalgae. They contain no nucleus, no chloroplasts, and have a different gene structure. There are approximately 2,000 species of cyanobacteria, which occur in many habitats. Although this group is distinguished by having members that can assimilate atmospheric N (thus eliminating the need to provide fixed N to the cells), no member of this class produces significant quantities of storage lipid; therefore, this group was not deemed useful to the ASP.

Close-Out Report

2. Sheehan, J. et al. (1998). DOE Close-Out Report.

Why Cyanobacteria?

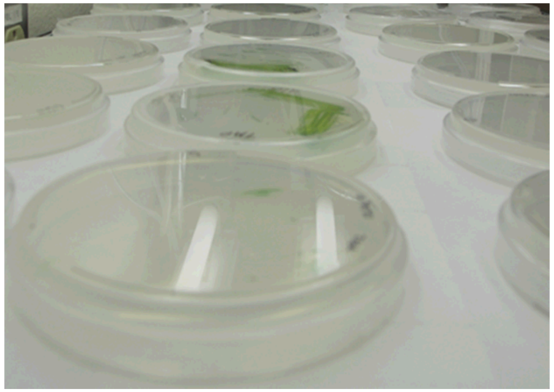
Advantages of Cyanobacteria for Fuel Production

Desirable strain traits

- Easily transformed
- Homologous recombination – targeted genome integration
- 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)



Paradigm shift in algal biofuels from lipid productivity to biomass productivity.

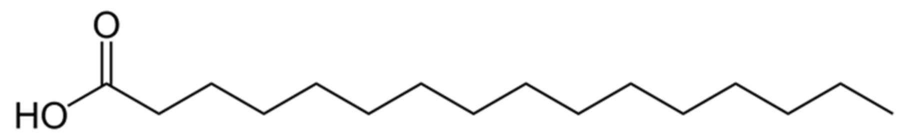
Overview

- Engineering cyanobacterial production of a biodiesel precursor
- Biofuel toxicity and potential solutions
- Development of a cyanobacterial chassis

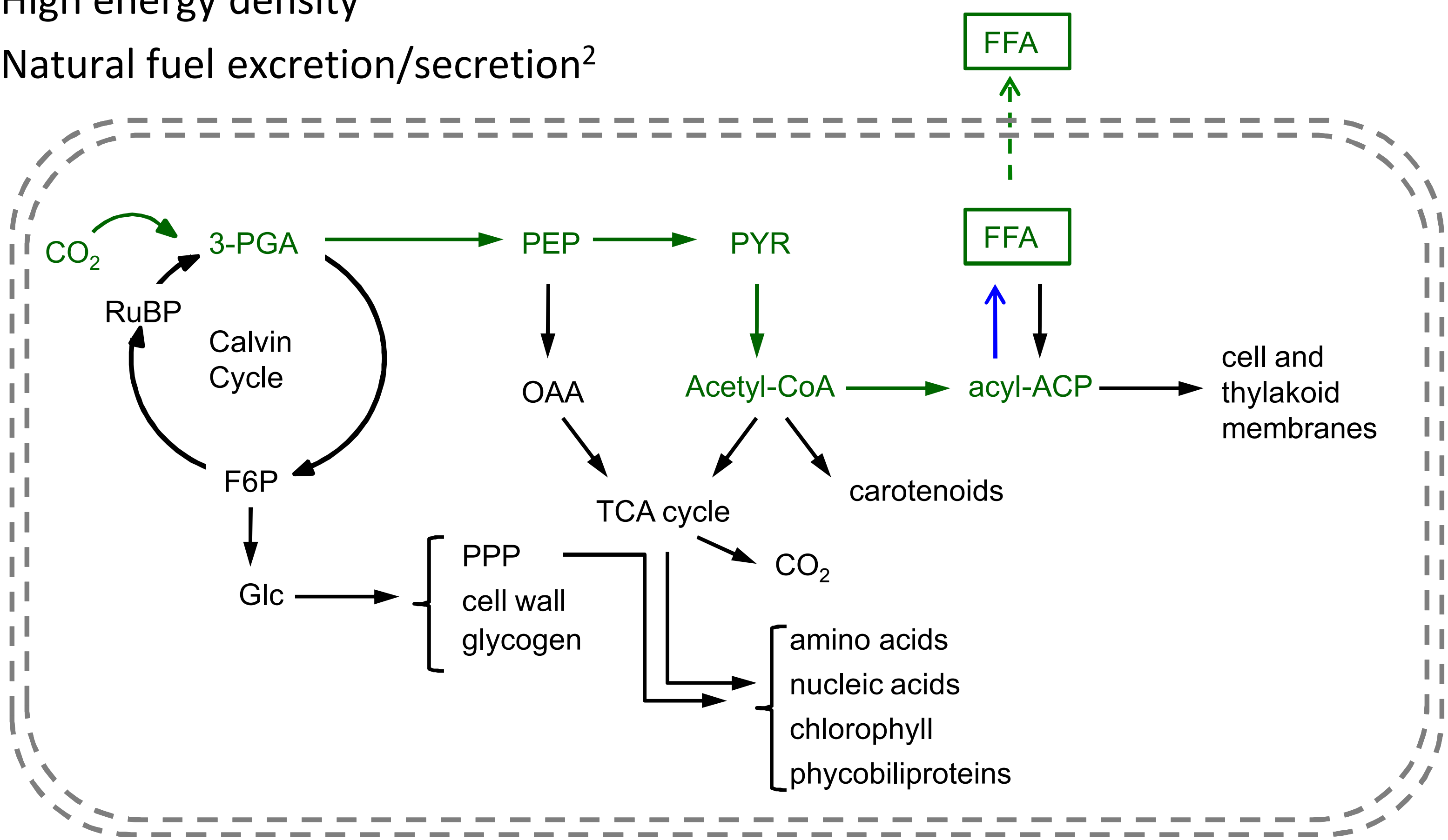
Target Fuel: Free Fatty Acids (FFA)

Desirable Product Characteristics

- Photoautotrophic growth
- Naturally produced biomolecule
- High energy density
- Natural fuel excretion/secretion[2]

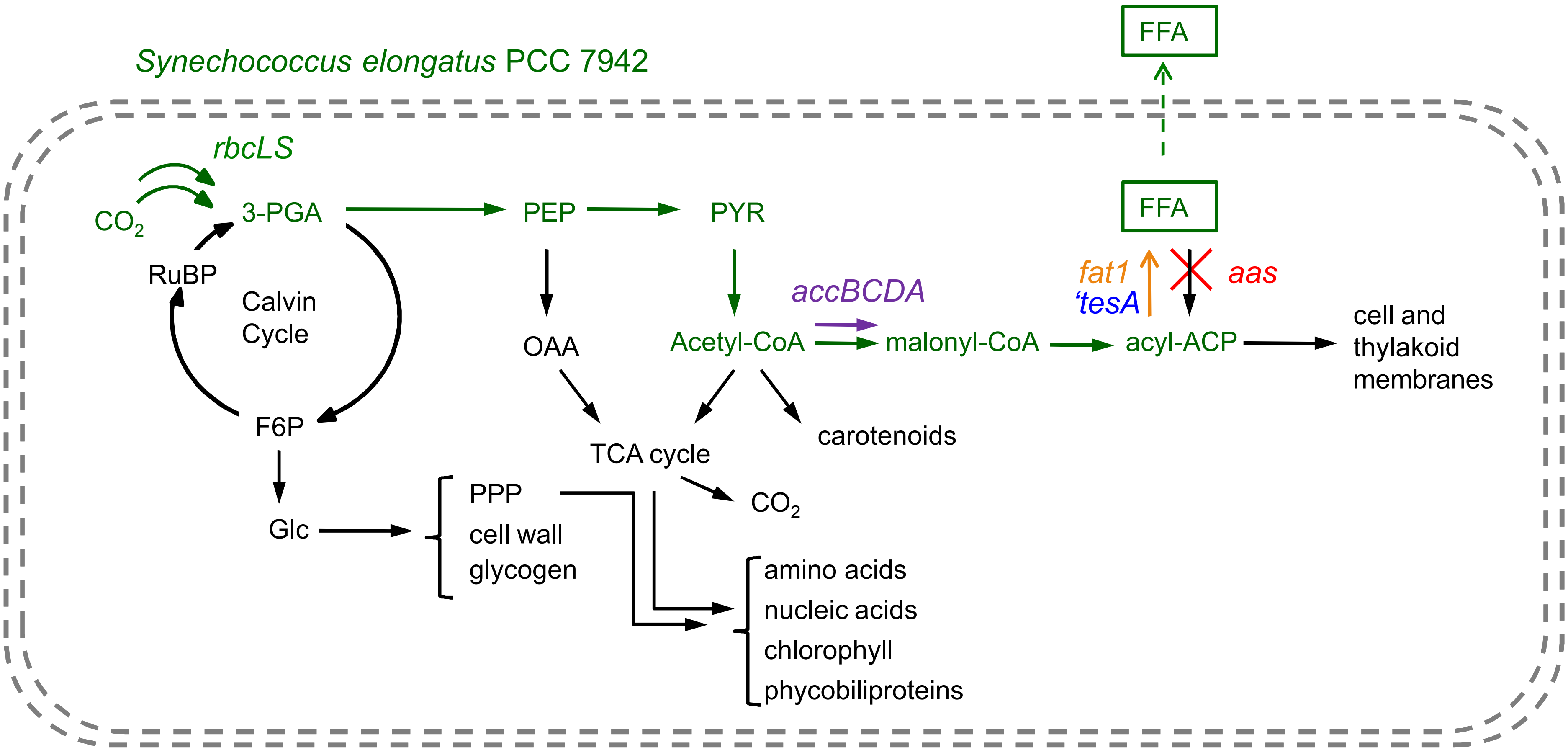


FFA: hexadecanoic acid



2. Kaczmarzyk D, Fulda M. (2010) Plant Physiology.

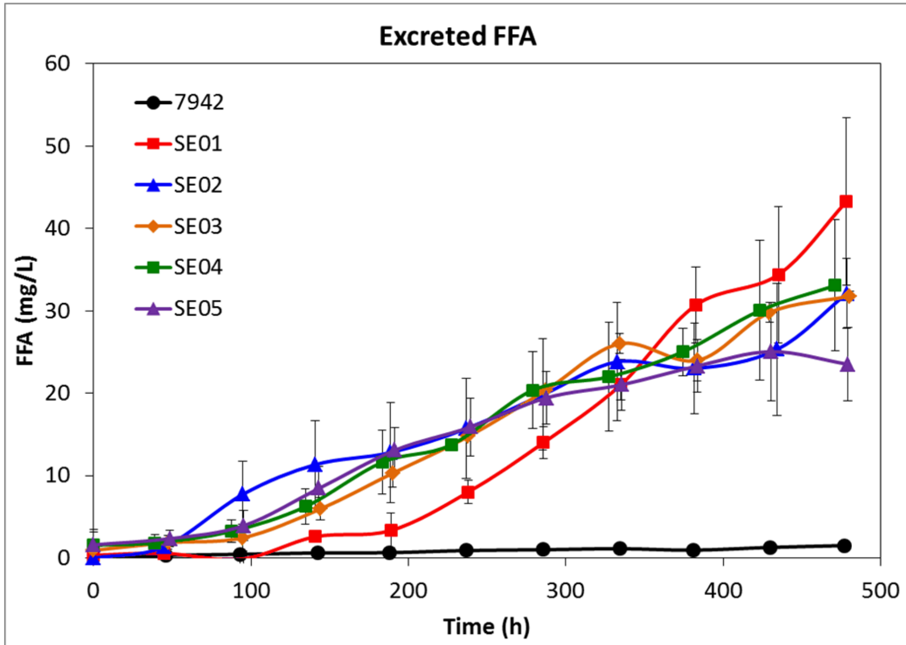
Genetic Engineering of Cyanobacteria to Produce FFA



7942: wild type; SE01: Δ*aas*; SE02: Δ*aas*, *'tesA*; SE03: Δ*aas*, *fat1*; SE04: Δ*aas*, *fat1*, *rbcLS*; SE05: Δ*aas*, *fat1*, *rbcLS*, *accBCDA*

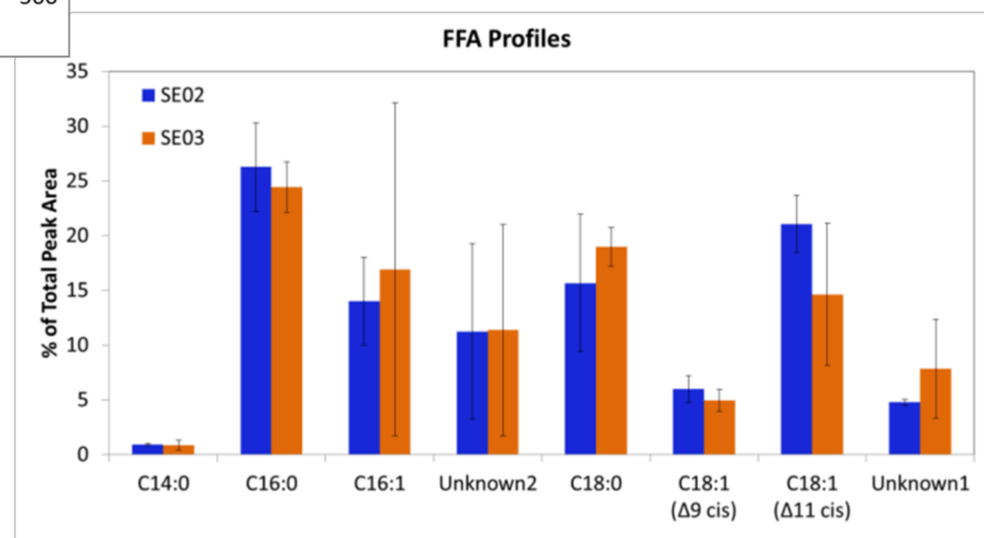
- *aas* – acyl-ACP synthetase / long-chain-fatty-acid CoA ligase
- *'tesA* – truncated thioesterase from *Escherichia coli*
- *fat1* – acyl-ACP thioesterase from *Chlamydomonas reinhardtii*
- *rbcLS* – native RuBisCO
- *accBCDA* – multi-subunit acetyl-CoA carboxylase from *C. reinhardtii* (chloroplast associated)

FFA Production in Engineered 7942 Strains



- All engineered strains produce and excrete FFA
- Without thioesterase expression, FFA only accumulate during stationary phase
- Despite targeting rate-limiting steps, the rate of FFA production is not improved

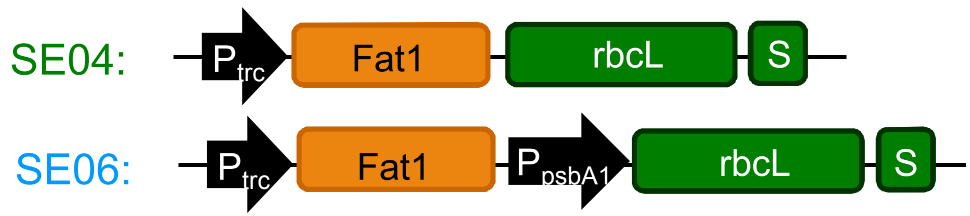
- Primarily C16 and C18 saturated and monounsaturated FFA are produced
- Both thioesterases (*E. coli* and *C. reinhardtii*) produce similar chemical profiles for excreted FFA

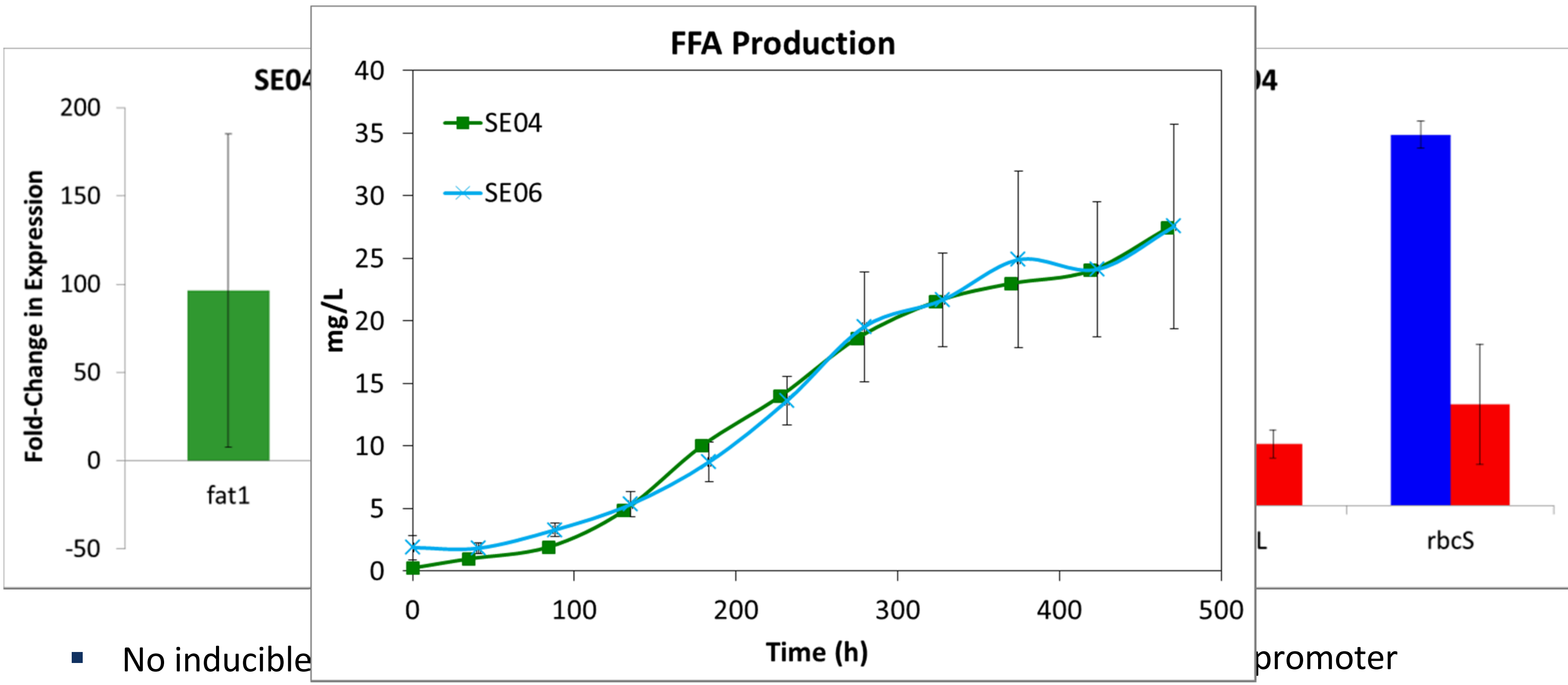


7942: wild type; SE01: Δ*aas*; SE02: Δ*aas*, *'tesA*; SE03: Δ*aas*, *fat1*; SE04: Δ*aas*, *fat1*, *rbcLS*; SE05: Δ*aas*, *fat1*, *rbcLS*, *accBCDA* 1

Ruffing AM, Jones HDT. 2012. *Physiological effects of free fatty acid production in genetically engineered Synechococcus elongatus PCC 7942*. Biotech. Bioeng. 109 (9): 2190-9.

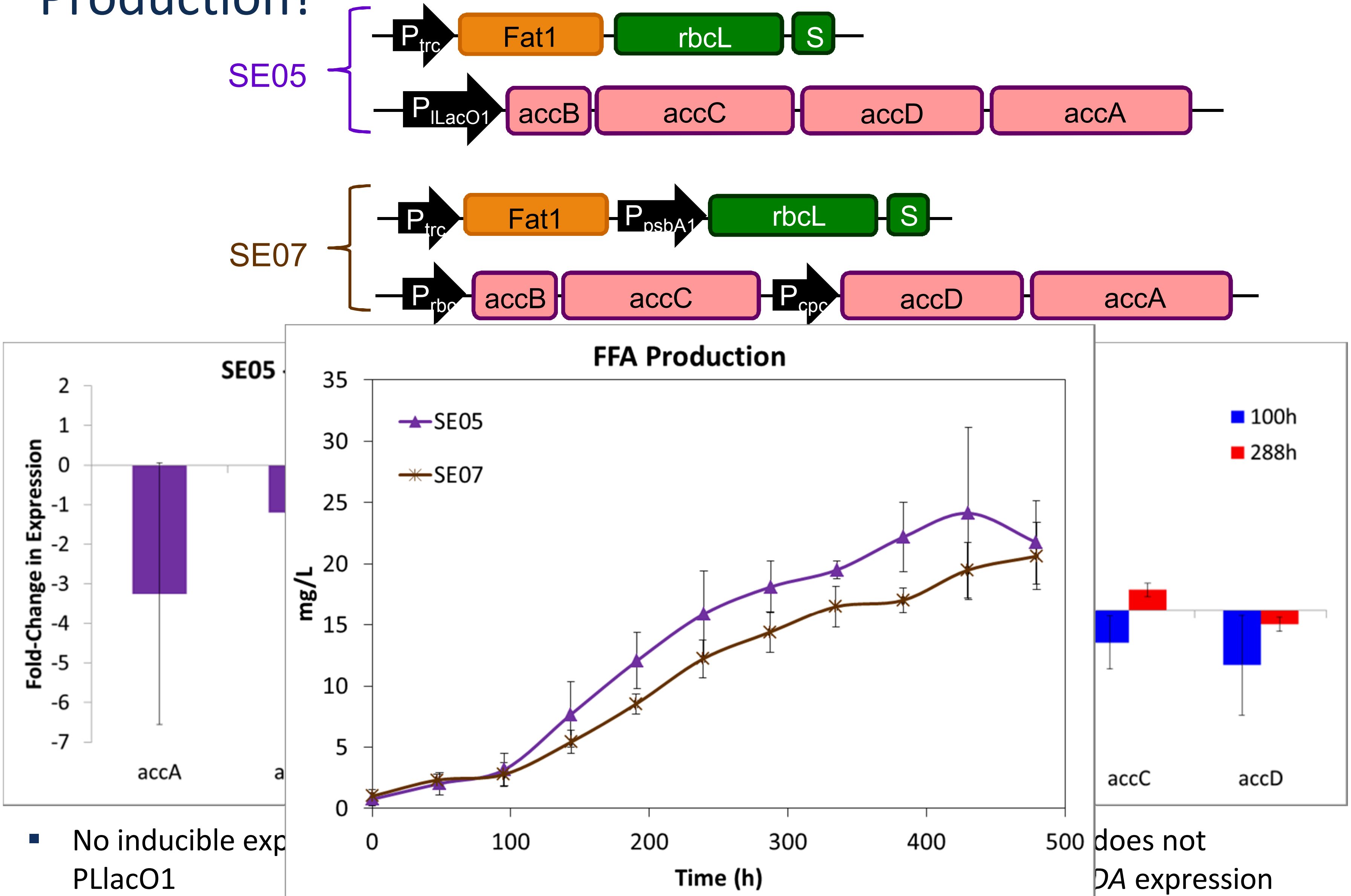
Does Increasing Gene Expression Improve FFA Production?





- No inducible … promoter improves *rbcLS* expression

Does Increasing Gene Expression Improve FFA Production?



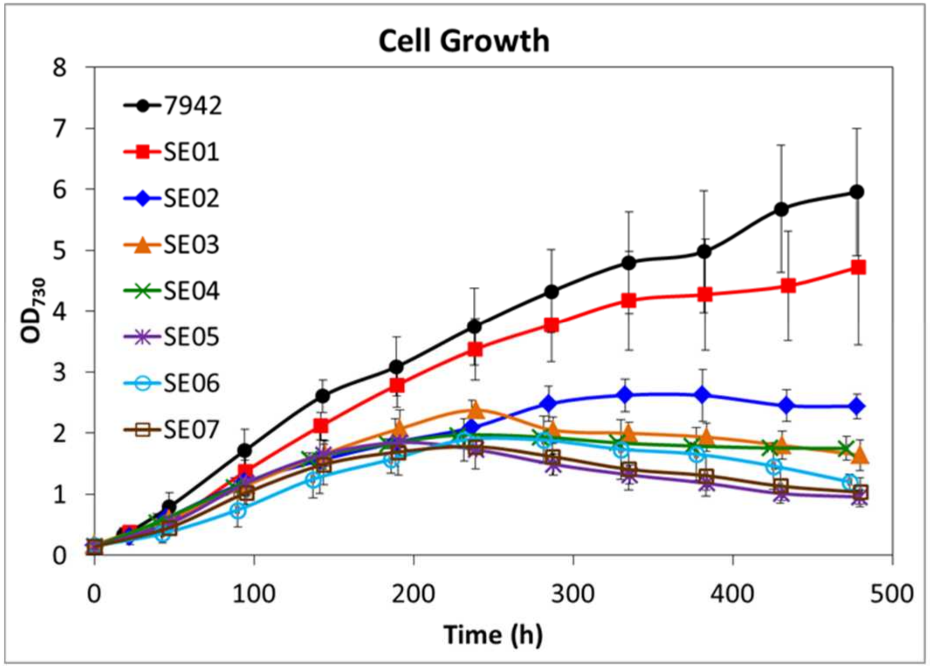
- No inducible exp… PLlacO1 … does not … DA expression

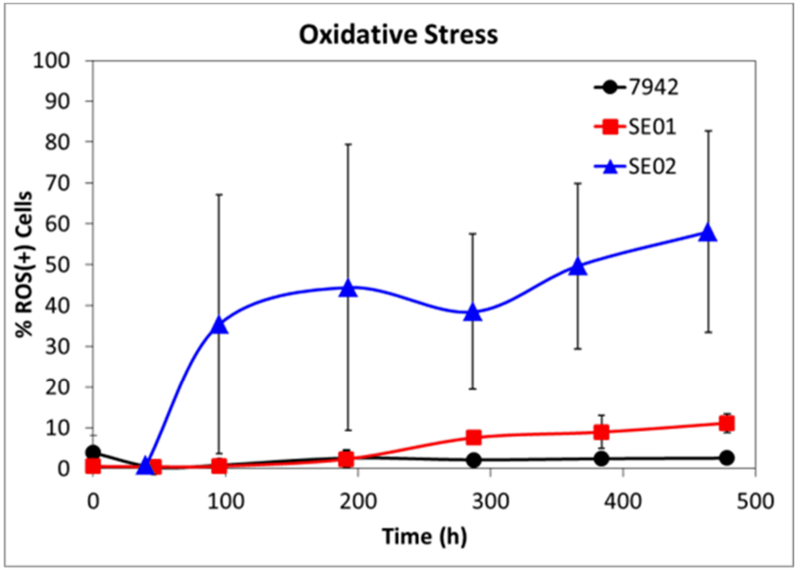
Overview

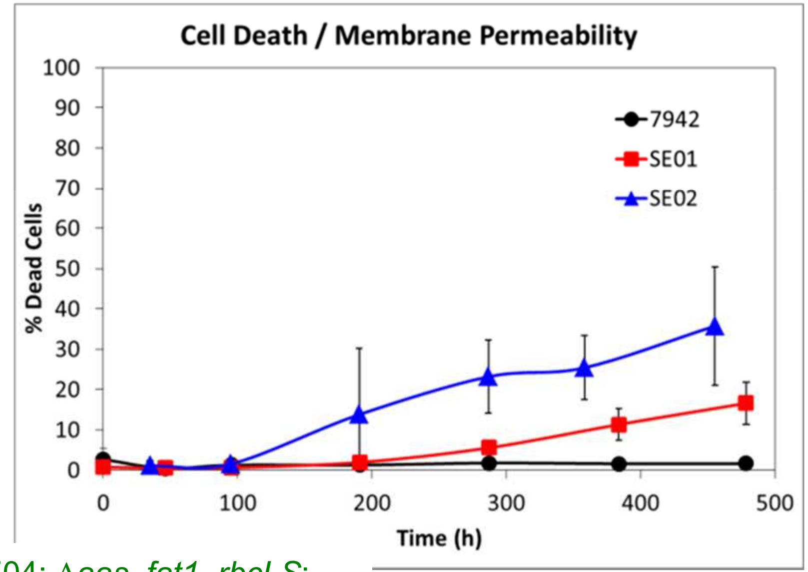
- Engineering cyanobacterial production of a biodiesel precursor
  - Demonstrated proof-of-concept
  - What is limiting FFA production in 7942?
- Biofuel toxicity and potential solutions
- Development of a cyanobacterial chassis

Physiological Effects: Growth, Stress, and Cell Death

- Final cell concentration reduced by more than 80% in SE05 and SE07
- FFA-producing strains have elevated levels of reactive oxygen species (ROS) and increased cell death / membrane permeability







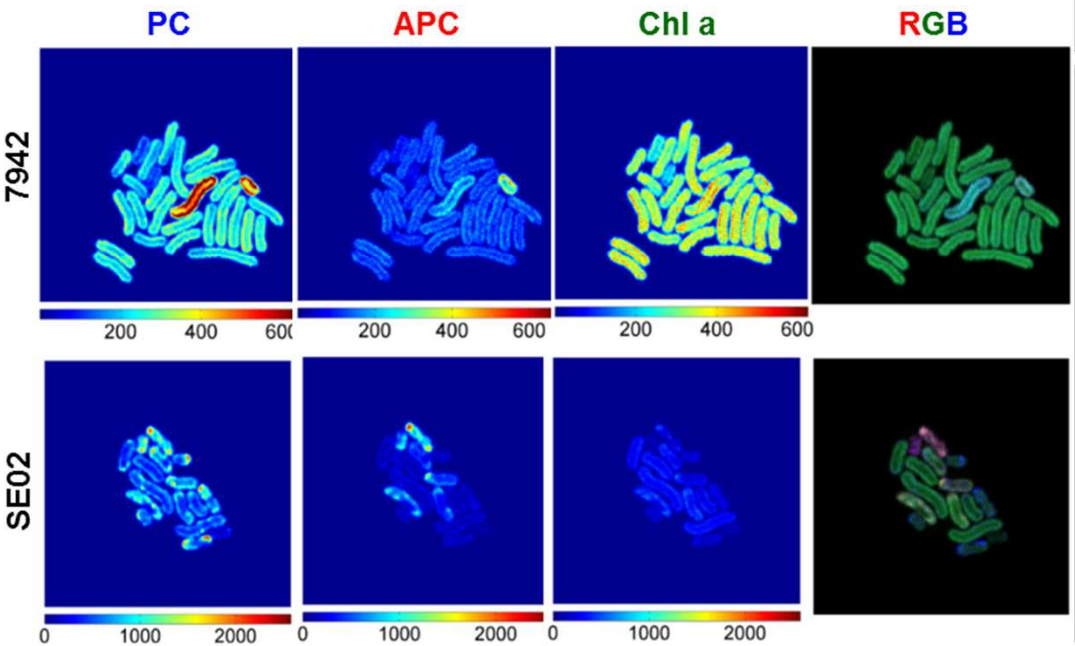
7942: wild type; SE01: Δ*aas*; SE02: Δ*aas, 'tesA*; SE03: Δ*aas, fat1*; SE04: Δ*aas, fat1*, *rbcLS*; SE05: Δ*aas, fat1*, *rbcLS*, *accBCDA* ; SE06: Δ*aas,* Fat1, P_{psbAI} *rbcLS;* SE07: Δ*aas,* Fat1, P_{psbAI} *rbcLS,* P_{rbc} *accBC* P_{cpc} *accDA*

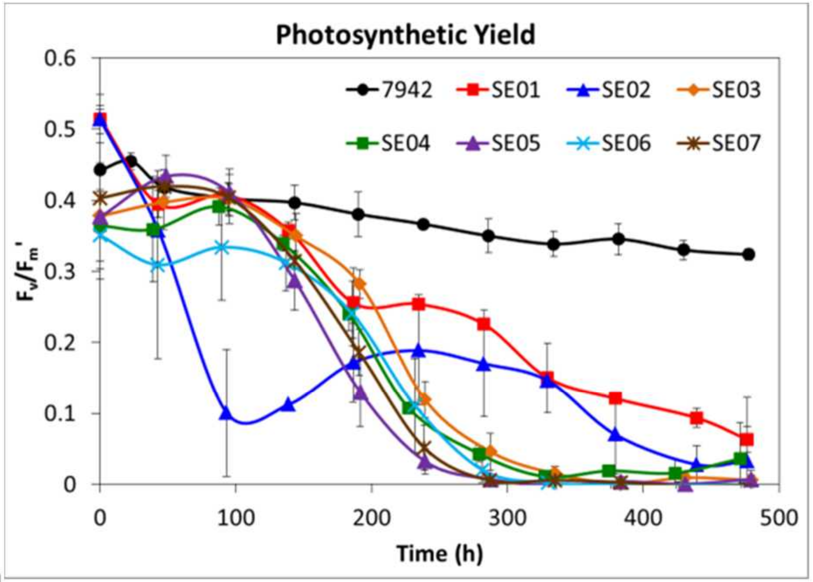
Ruffing AM, Jones HDT. 2012. *Physiological effects of free fatty acid production in genetically engineered Synechococcus elongatus PCC 7942.* Biotech. Bioeng. 109 (9): 2190-9.

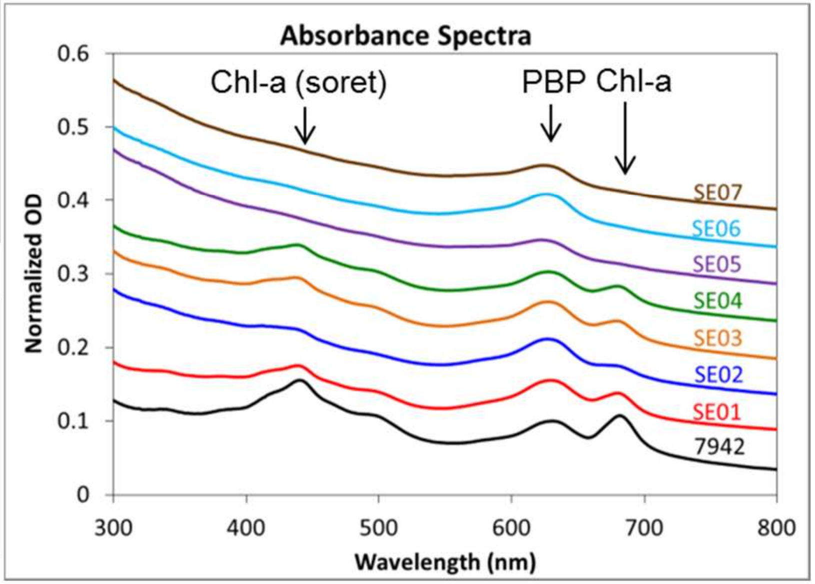
Photosynthetic Effects

Sandia National Laboratories

- Photosynthetic yield drops to zero in FFA-producing strains
- Bulk absorbance measurements indicate a selective degradation of chlorophyll-a pigment
- Hyperspectral confocal fluorescence microscopy shows photosynthetic pigments are aggregating at the cell poles in the engineered strain SE02







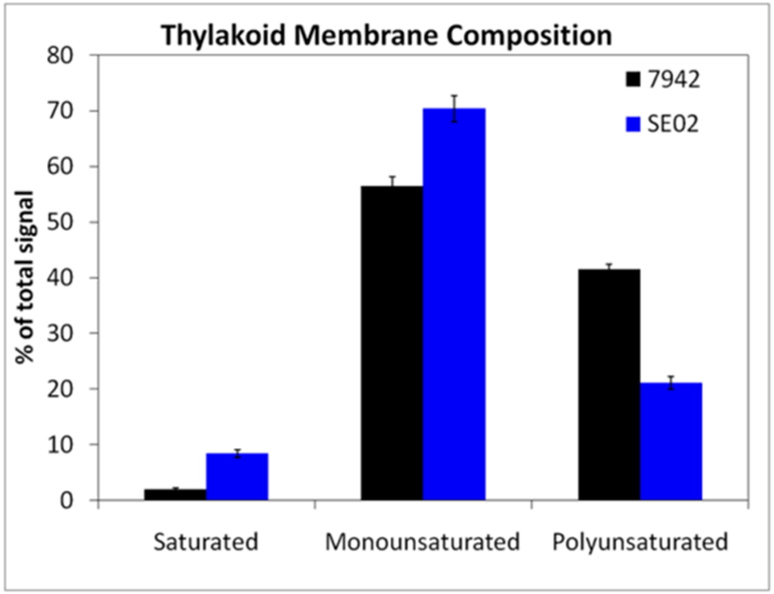
7942: wild type; SE01: Δ*aas*; SE02: Δ*aas, ‘tesA*; SE03: Δ*aas, fat1*; SE04: Δ*aas, fat1*, *rbcLS*;

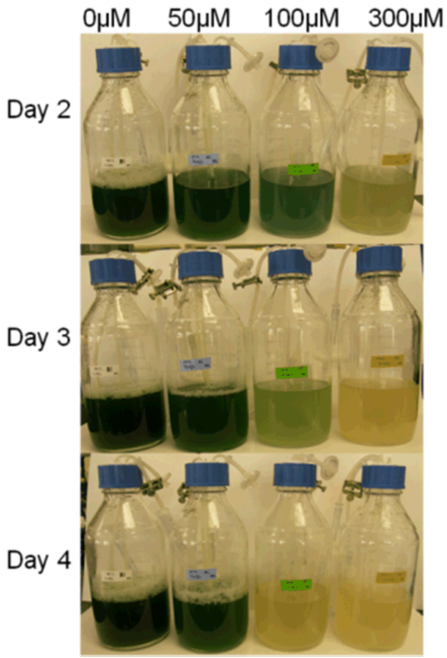
SE05: Δ*aas, fat1*, *rbcLS*, *accBCDA* ; SE06: Δ*aas,* Fat1, P_{psbAI} *rbcLS;* SE07: Δ*aas,* Fat1, P_{psbAI} *rbcLS,* P_{rbc} *accBC* P_{cpc} *accDA*

Possible Mechanisms of FFA Effects

Mechanism 1: Engineered strains have altered membrane composition

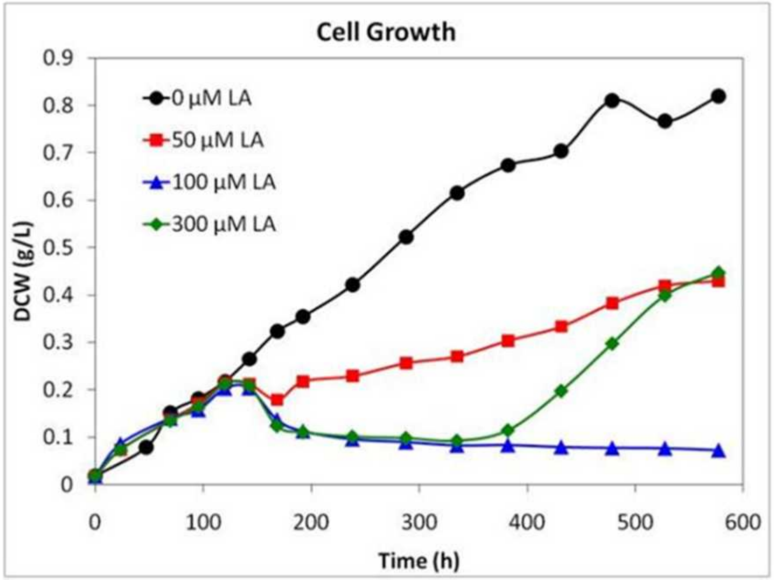
- Increased levels of saturated FA and lower levels of polyunsaturated FA in thylakoid membranes
- Leads to increased membrane viscosity and potential effect on phycobilisome attachment





Mechanism 2: FFA toxicity

- Exogenous saturated FFA has no effect on cell physiology
- Unsaturated FFA (linolenic acid - LA) oxidize into a variety of compounds, including toxic hydroperoxides



Ruffing AM, Jones HDT. 2012. *Physiological effects of free fatty acid production in genetically engineered Synechococcus elongatus PCC 7942*. Biotech. Bioeng. 109 (9): 2190-9.

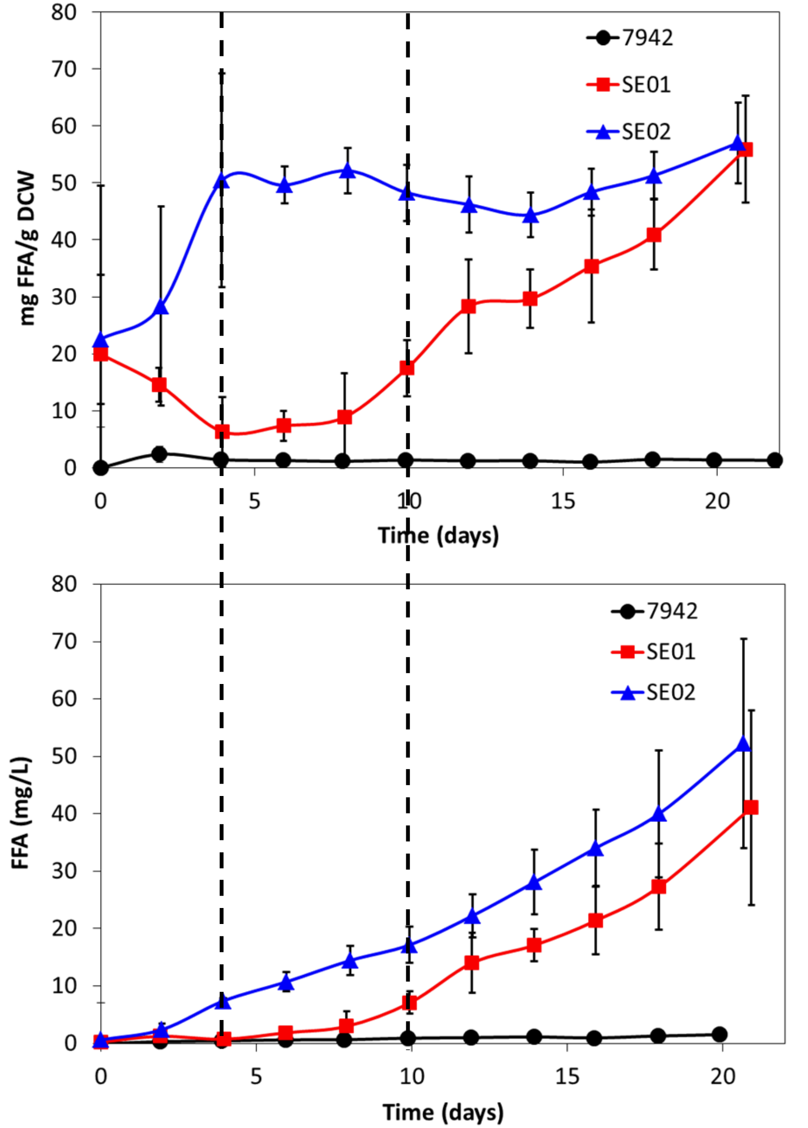
Can *S. elongatus* 7942 be engineered to overcome these effects?

RNA-seq to identify genetic response to FFA production

- 3 strains: 7942, SE01, SE02
- 2 time points: day 4, day 10
- 3 biological replicates

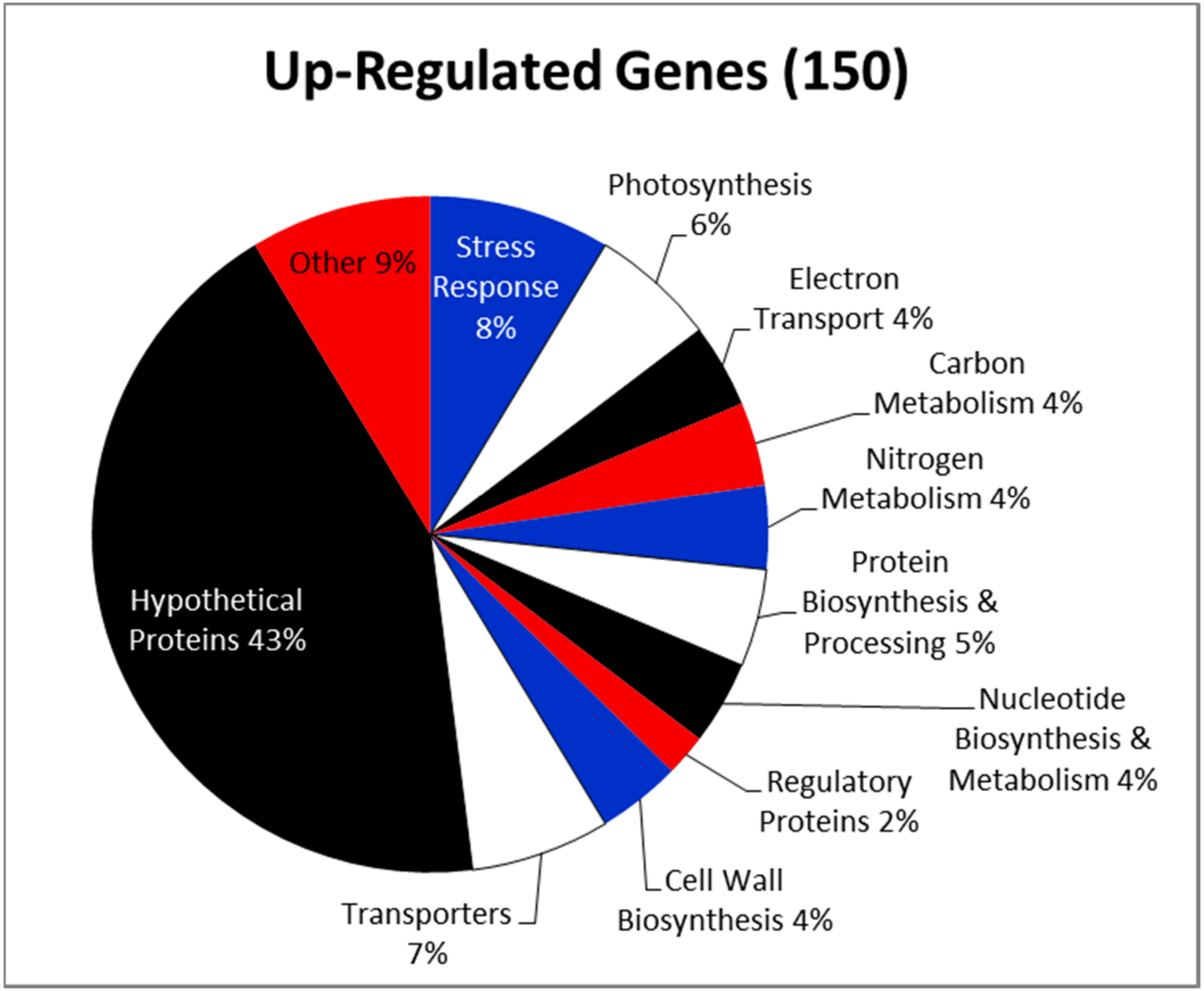
Differential gene expression comparisons:

	Low FFA	High FFA
A	SE01, day 4	SE02, day 4
B	7942, day 4	SE02, day 4
C	7942, day 10	SE01, day 10
D	7942, day 10	SE02, day 10
E	SE01, day 4	SE01, day 10
F	SE02, day 4	SE02, day 10



7942: wild type; SE01: Δ*aas;* SE02: Δ*aas, 'tesA*

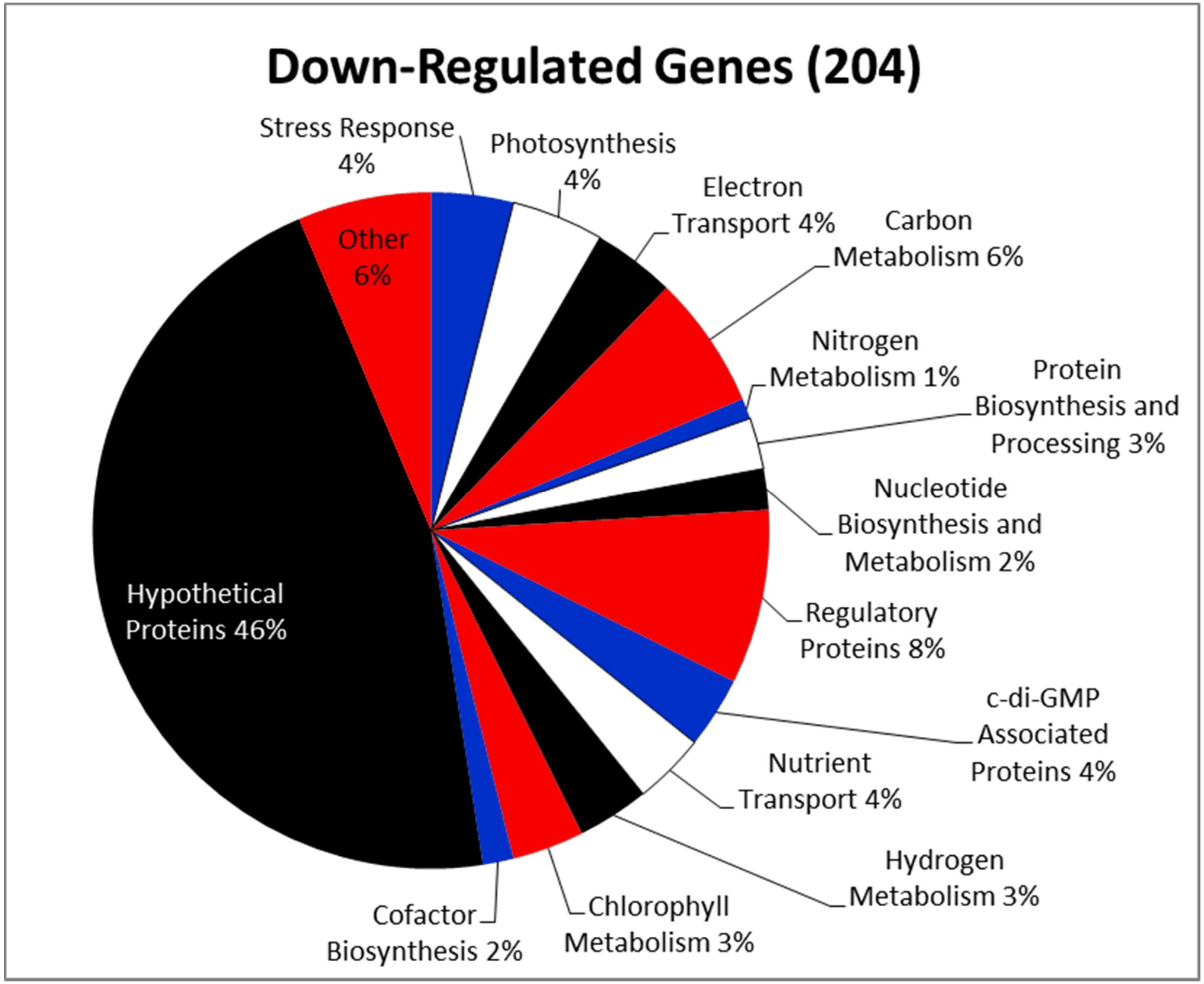
RNA-seq Analysis of FFA-Producing Cyanobacteria



Differential Gene Expression: Fold change > 2, p-value < 0.05.

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

RNA-seq Analysis of FFA-Producing Cyanobacteria



Differential Gene Expression: Fold change < -2, p-value < 0.05.

Identifying Targets for Improved FFA Production

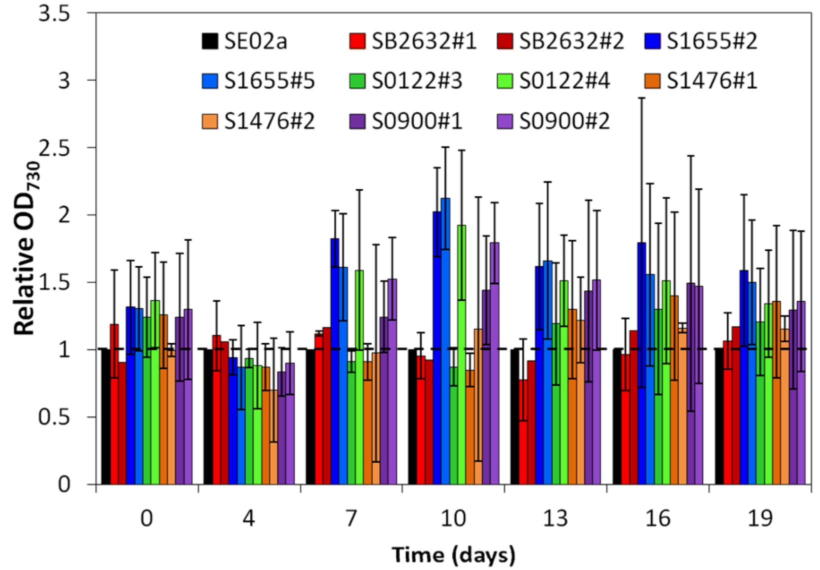
Locus	Product	Average FC	Targeted Mutagenesis
Hypothetical Proteins			
Synpcc7942_0444	hypothetical protein	3.27	Knockout
Synpcc7942_1561	hypothetical protein	2.67	Knockout
Synpcc7942_1023	hypothetical protein	2.15	Knockout
Synpcc7942_1476	hypothetical protein	-4.59	Overexpression
Synpcc7942_B2645	hypothetical protein	-7.35	Overexpression
Synpcc7942_1655	hypothetical protein	-2.98	Overexpression
Synpcc7942_0900	hypothetical protein	-2.92	Overexpression
Synpcc7942_B2632	hypothetical protein	-2.68	Overexpression
Synpcc7942_0122	hypothetical protein	-2.53	Overexpression
Synpcc7942_1845	hypothetical protein	-2.28	Overexpression
ROS-Degrading Proteins			
Synpcc7942_1214	glutathione peroxidase	2.63	Overexpression
Synpcc7942_0801	superoxide dismutase	2.56	Overexpression
Synpcc7942_0437	glutathione peroxidase	2.54	Overexpression
Synpcc7942_1656	catalase/peroxidase HPI	-2.38	Overexpression
Potential FFA Exporters			
Synpcc7942_2175	transport system substrate-binding protein	2.99	Knockout
Synpcc7942_1224	ABC-transporter membrane fusion protein	2.74	Knockout
Synpcc7942_1464	porin	2.33	Knockout
Synpcc7942_1607	porin; major outer membrane protein	2.16	Knockout

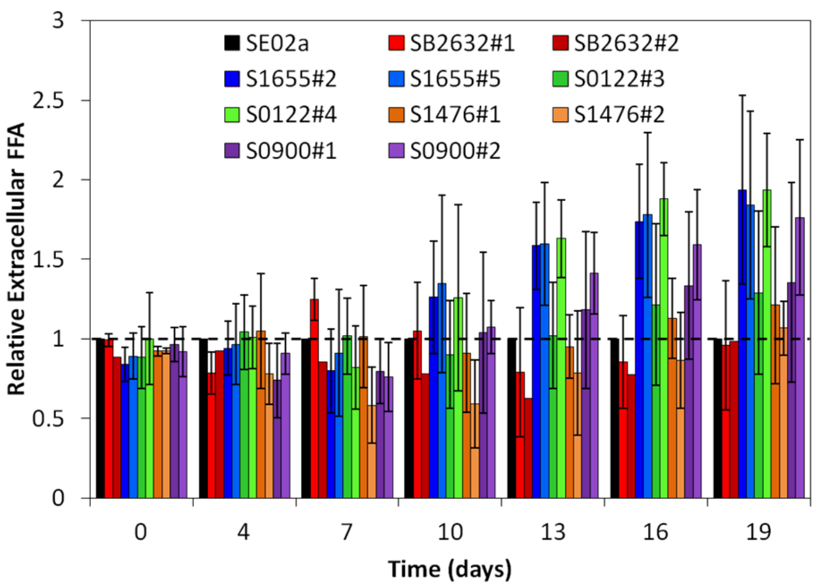
Ruffing AM. 2013. *RNA-Seq analysis and targeted mutagenesis for improved free fatty acid production in an engineered cyanobacterium.* Biotech. for Biofuels 6:113.

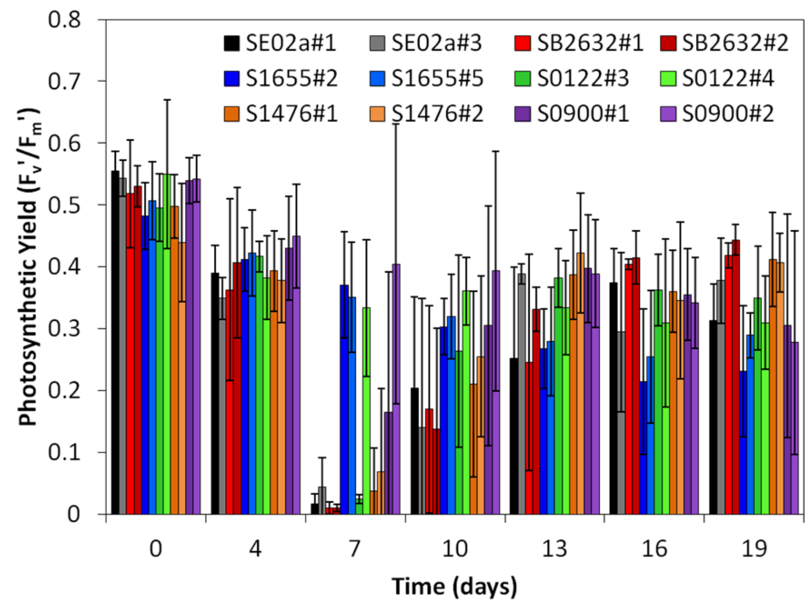
Hypothetical Protein Overexpression Mutants

Improved cell growth, photosynthetic yield, and FFA production:

- S1655
- S0122 - EAL domain, putative diguanylate phosphodiesterase
- S0900 - glutamine synthetase





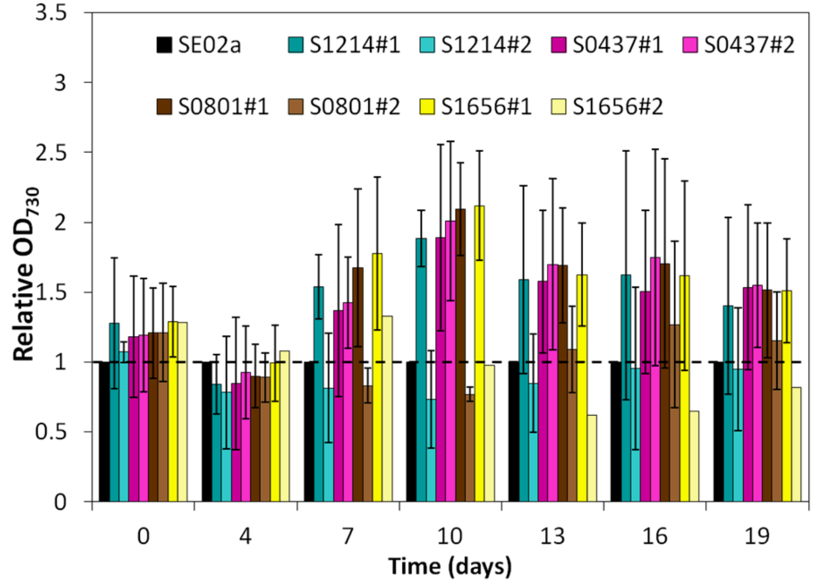


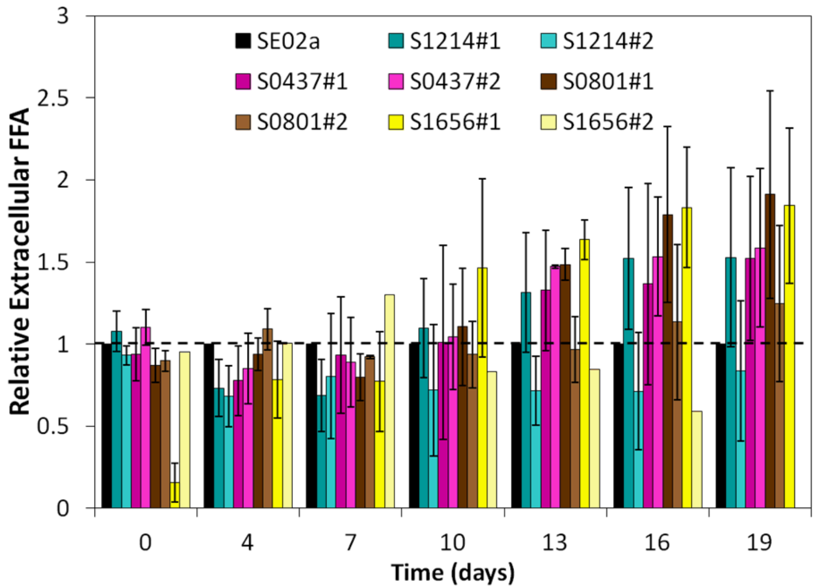
Ruffing AM. 2013. *RNA-Seq analysis and targeted mutagenesis for improved free fatty acid production in an engineered cyanobacterium.* Biotech. for Biofuels 6:113.

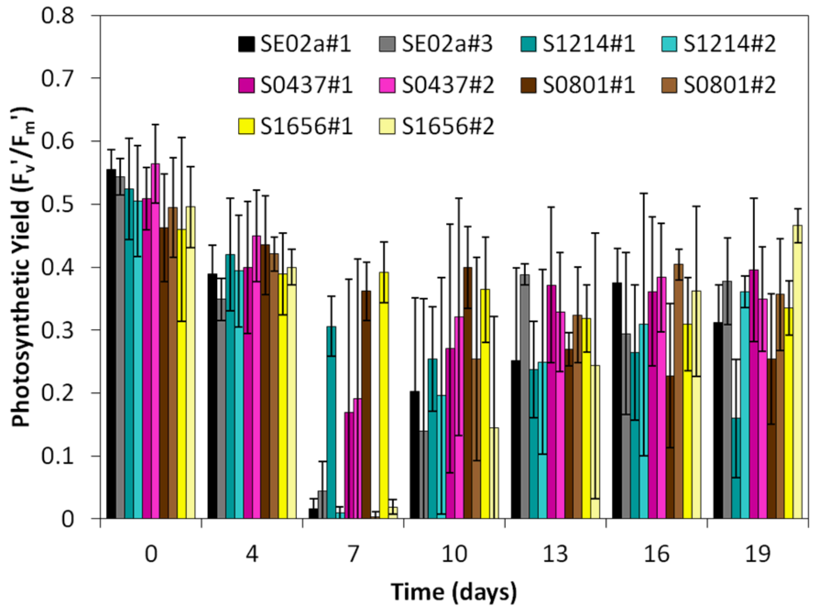
ROS-Degrading Protein Overexpression Mutants

Improved cell growth, photosynthetic yield, and FFA production:

- S1214 - glutathione peroxidase
- S0437 - glutathione peroxidase
- S0801 - superoxide dismutase
- S1656 - catalase/peroxidase





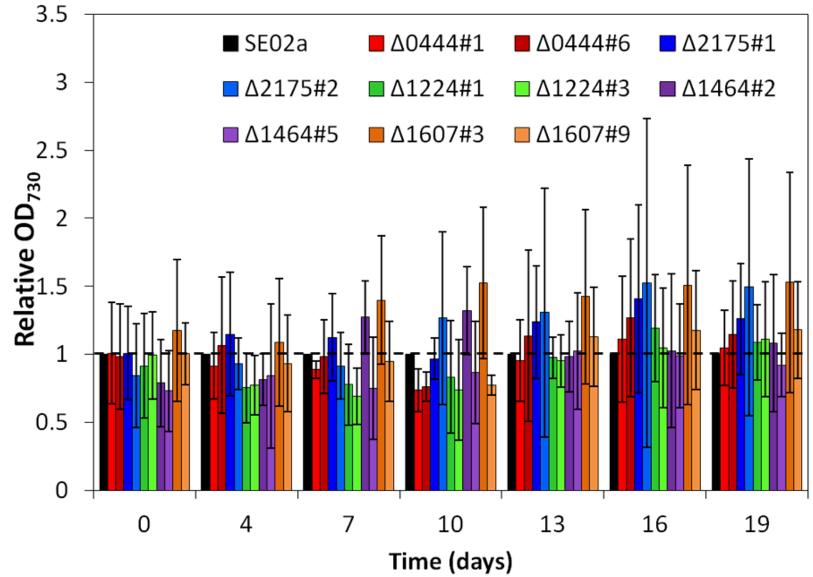


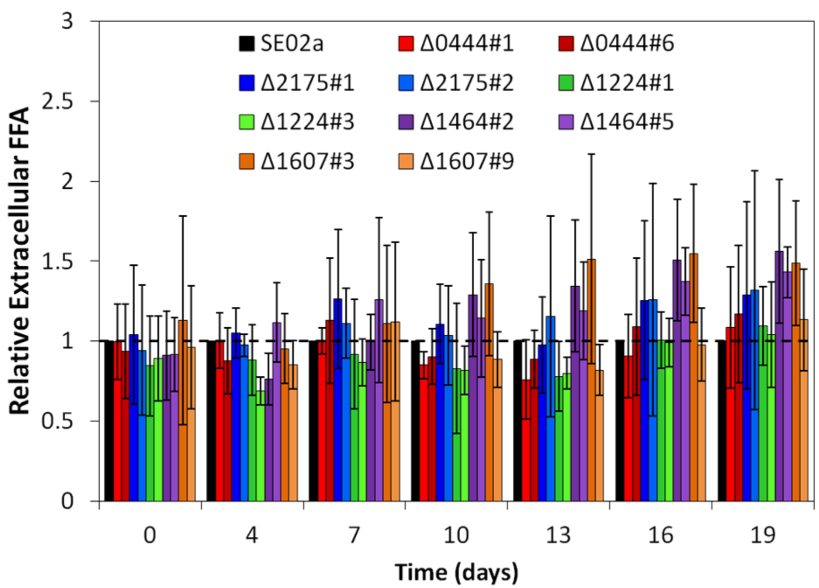
Ruffing AM. 2013. *RNA-Seq analysis and targeted mutagenesis for improved free fatty acid production in an engineered cyanobacterium.* Biotech. for Biofuels 6:113.

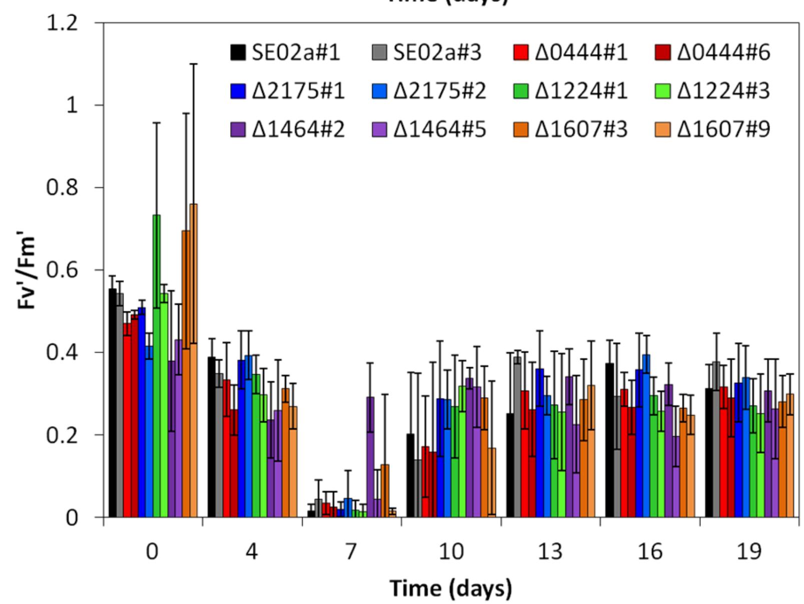
Knockout Mutants (Candidate FFA Export Proteins)

Improved photosynthetic yield and FFA production, but no increase in cell growth:

- Δ1464 - porin
- Δ1607 - porin; major outer membrane protein

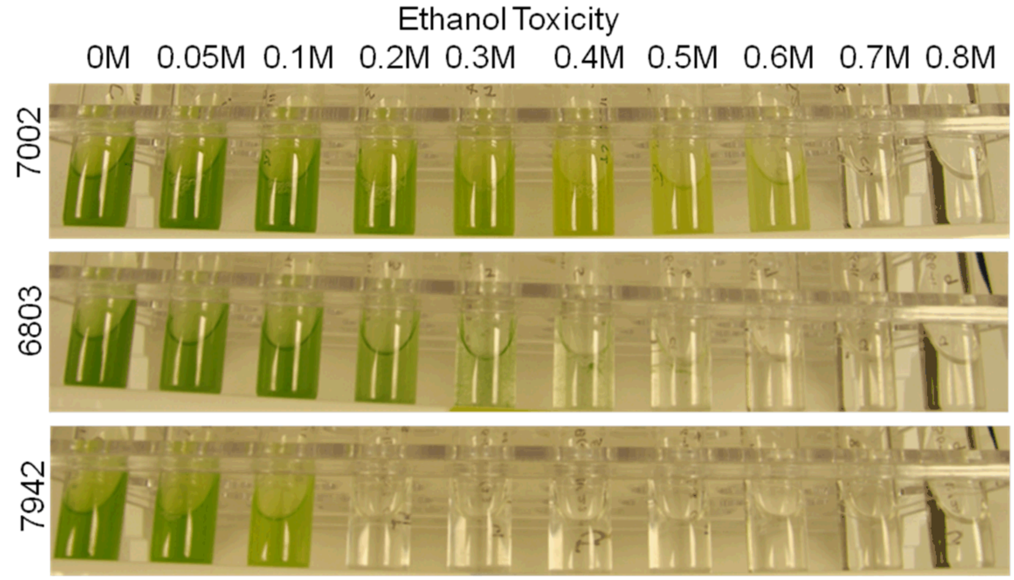






Does host selection effect FFA production? *Synechococcus* sp. PCC 7002

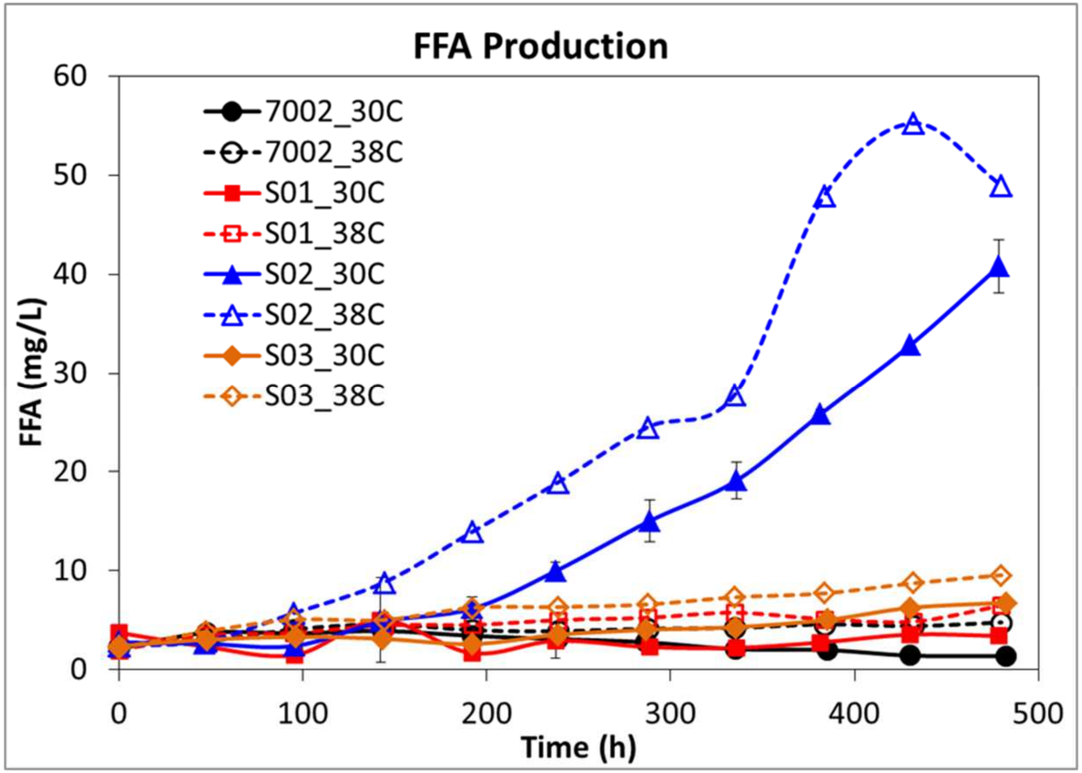
- Model cyanobacterium
  - Genetic tools available
  - Genome sequence
- Salt tolerance
- High light tolerance
- Biofuel tolerance



Genetic modifications	*S. elongatus* 7942	*Synechococcus* sp. 7002
Δaas/fadD	SE01	S01
Δaas/fadD, ‘tesA	SE02	S02
Δaas/fadD, Fat1	SE03	S03
Δaas, Fat1 (SE04) or *‘tesA* (S05), *rbcLS*	SE04	S05
Δaas, Fat1, rbcLS, accBCDA	SE05	S06
Δaas, Fat1 (SE06) or *‘tesA* (S07), P_{psbAI} *rbcLS*	SE06	S07
Δaas, Fat1, P_{psbAI} *rbcLS*, P_{rbc} *accBC* P_{cpc} *accDA*	SE07	

Ruffing AM. 2014. Improved free fatty acid production in cyanobacteria with Synechococcus sp. PCC 7002 as host. Frontiers in Bioeng. & Biotech. 2:17.

FFA Production in 7002 Strains



7002: wild type

S01: Δ*fadD*

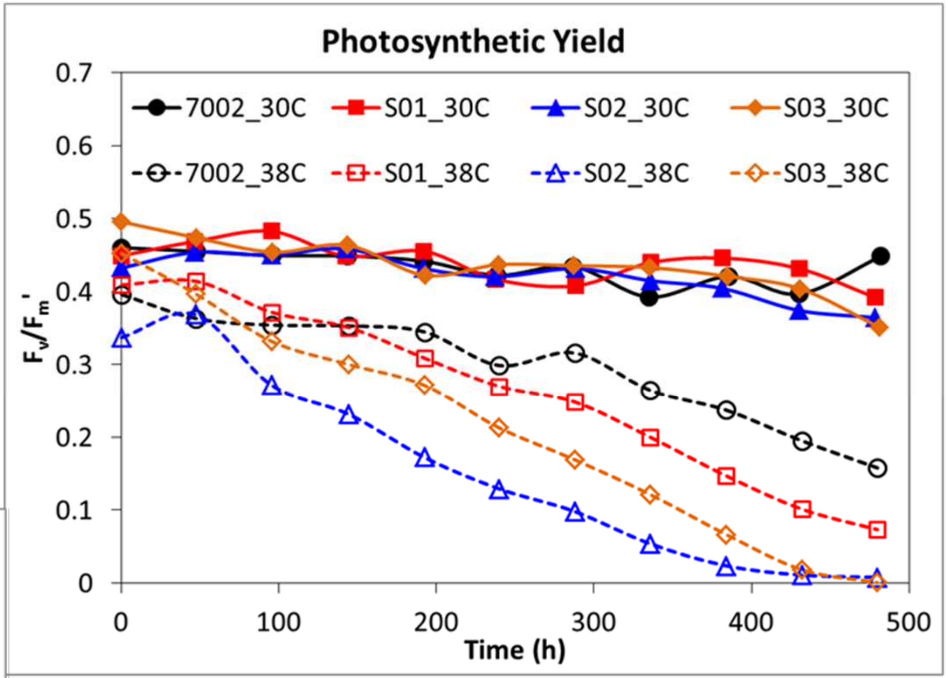
S02: Δ*fadD, 'tesA*

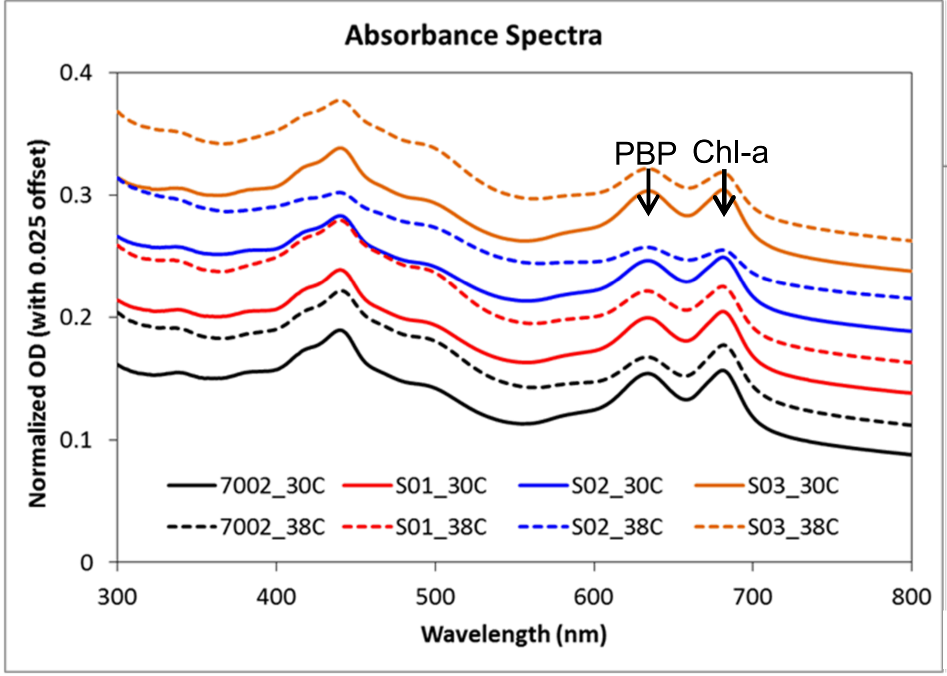
S03: Δ*fadD, fat1*

- FFA is produced and excreted by engineered 7002 strains
- 45–fold more FFA is produced using the *E. coli* thioesterase (*'tesA*, S02) compared to the *C. reinhardtii* acyl-ACP thioesterase (*fat1*, S03)
- The optimal growth temperature (38°C) leads to more FFA production compared to 30°C

Physiological Effects of FFA Production in 7002 Strains

- Photosynthetic yields (F_v'/F_m') remain constant at 30°C for the FFA-producing 7002 strains.
- At 38°C, there is a gradual decline in photosynthetic yield throughout FFA biosynthesis for all 7002 strains, yet this effect is most severe in the highest yielding FFA strain, S02.





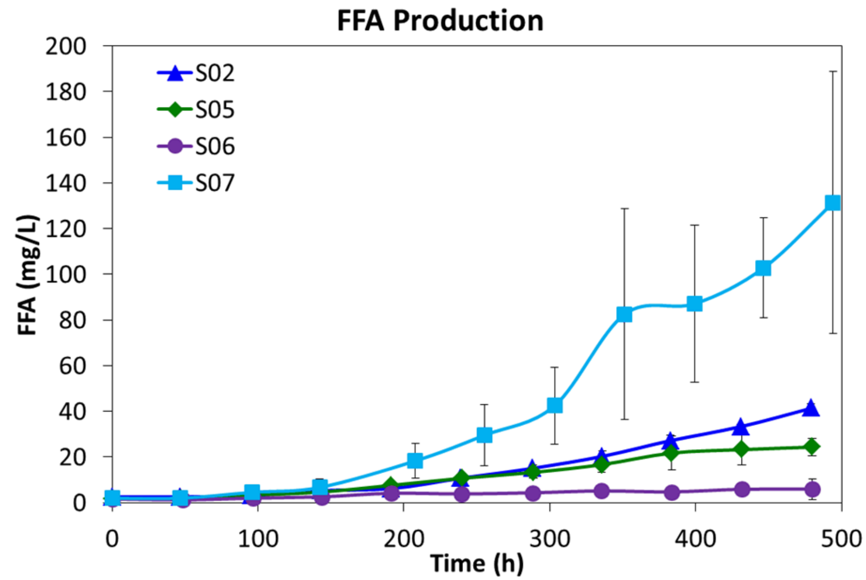
- No change in photosynthetic pigments for 7002 strains at 30°C.
- S02 shows degradation of both phycobiliprotein and Chl-a pigments at 38°C.
- This response differs from that of 7942, which showed selective degradation of Chl-a.

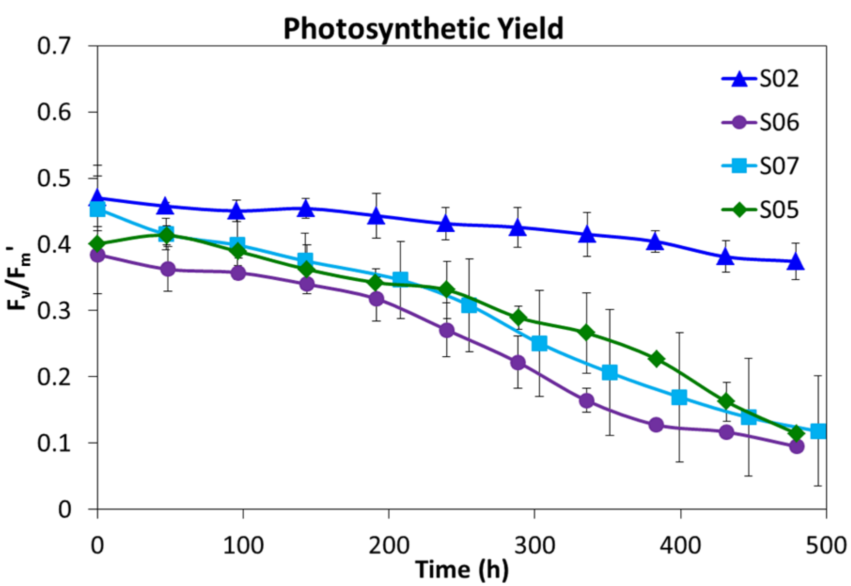
7002: wild type; S01: Δ*fadD;* S02: Δ*fadD, 'tesA;* S03: Δ*fadD, fat1*

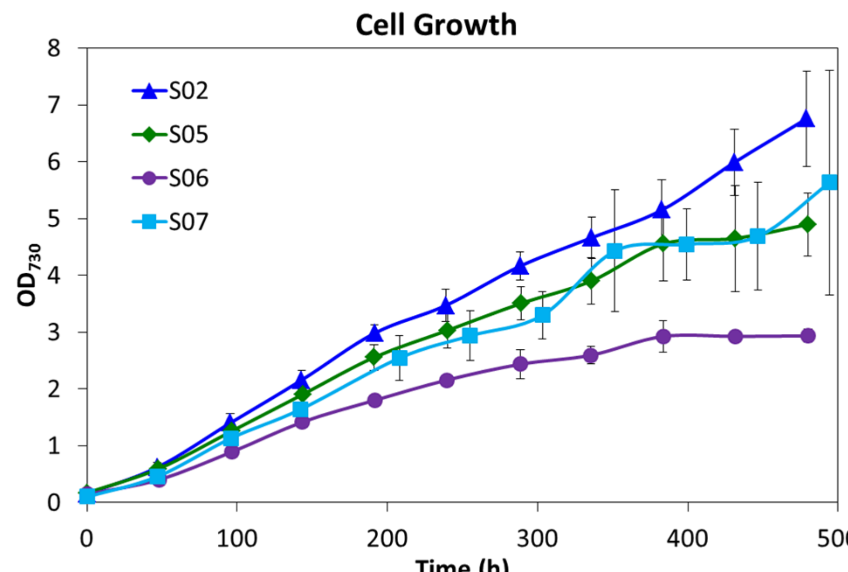
RuBisCO Overexpression Improves FFA Yield

Sandia National Laboratories

- High FFA production in S07 (*Δaas, tesA*, PpsbAI-*rbcLS*) compared to S05 (*Δaas, tesA, rbcLS*)
- S07 has decreased photosynthetic yields and a slight reduction in cell growth







S02: *Δaas, 'tesA*; S05: *Δaas, tesA, rbcLS*; S06: *Δaas, tesA, rbcLS, accBCDA;* S07: *Δaas, tesA*, PpsbAI-*rbcLS*

Overview

- Engineering cyanobacterial production of a biodiesel precursor
  - Demonstrated proof-of-concept
  - What is limiting FFA production in 7942?
- Biofuel toxicity and potential solutions
  - Genetic targets identified for reduced FFA toxicity
  - Cyanobacterial host selection is important
- Development of a cyanobacterial chassis

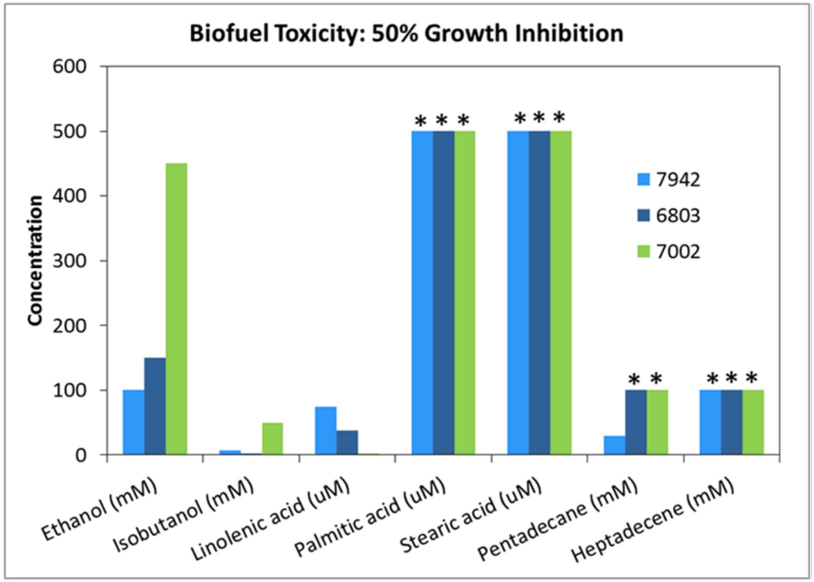
Cyanobacterial Biofuels vs. *E. coli* Biofuels

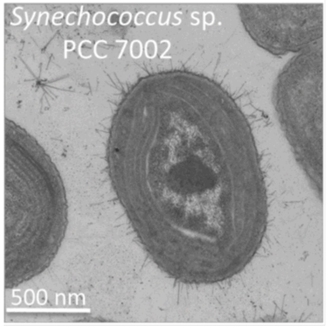
Fuel/ Precursor	LHV (kJ/mol)	Host	Productivity (mmol/L/h)	Energy Productivity (kJ/L/h)
Ethanol	1370	*Escherichia coli*	15 (Munjal 2012)	20.55
		Synechocystis sp. PCC 6803	0.013 (Dexter 2009)	0.018
Butanol	2670	*Escherichia coli*	0.024 (Atsumi 2008)	0.064
		Synechococcus elongatus PCC 7942	0.0017 (Lan 2011)	0.0045
FFA (hexadecanoic acid)	10,107	*Escherichia coli*	0.35 (Liu 2012)	3.55
		Synechococcus sp. PCC 7002	0.0011 (Ruffing 2014)	0.011

1 mol glucose → 2870 kJ

Synechococcus sp. PCC 7002 as a Cyanobacterial Chassis

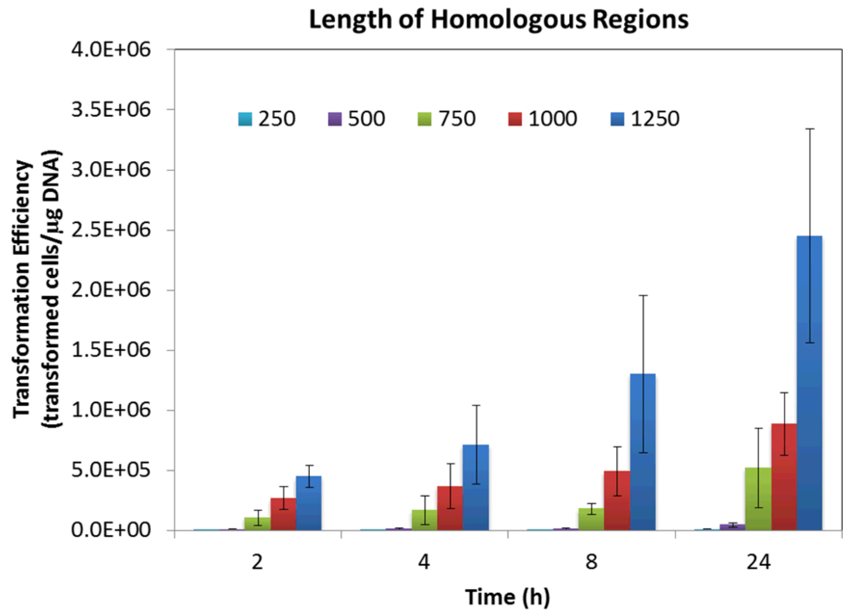
- Genome sequence available
- Fast growth rate
  - Doubling times as low as 2.6 h reported[3]
- Marine strain
  - Tolerates up to 3 M salt[4] (seawater ~ 0.5 M)
  - Can grow on non-freshwater sources
  - Tolerant to evaporation
- High light tolerance
  - Tolerates up to 2.5 x peak sunlight[5]
- Temperature tolerance
  - Can grow from 22 - 40°C[3]
  - Photobioreactors can reach high temperatures (40-45°C)
- More biofuel tolerant compared to model freshwater cyanobacteria[6]



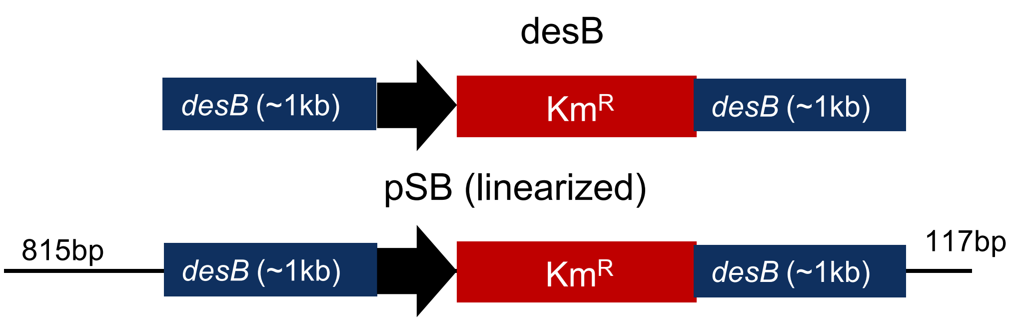


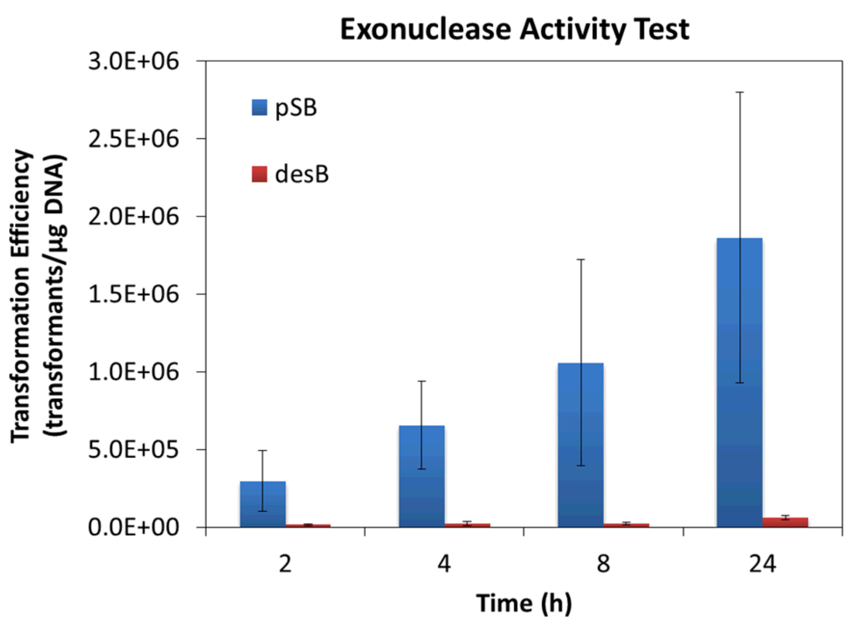
3. http://bioenergy.asu.edu/cb&p/abstracts/Bryant-abstract.html. 4. Ludwig M, Bryant DA. 2012 Frontiers in Microbiol. 3:354. 5. Nomura CT, Sakamoto T, Bryant DA. 2006. Arch. Microbiol. 185:471-479. 6. Ruffing AM, Trahan CA. 2014. Algal Research 5:121-132.

Improving Transformation of *Synechococcus* sp. PCC 7002



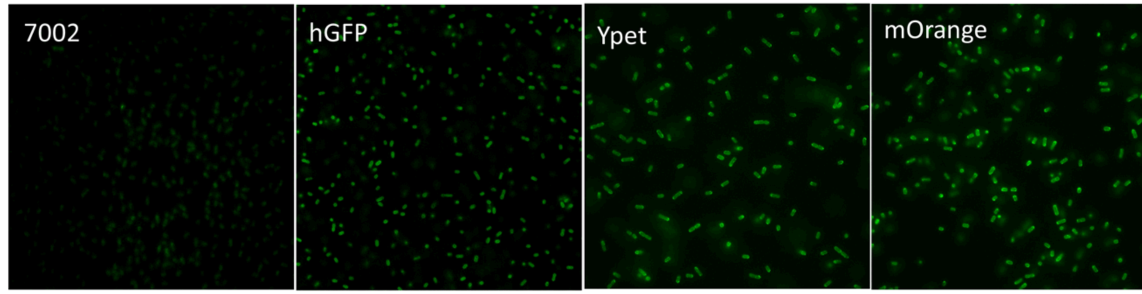
- Minimum length of homology arms = 250 bp
- Exonuclease activity detected in *Synechococcus* sp. PCC 7002
- Genome includes 3 predicted exonucleases (two are single-stranded specific)
    - Candidate exonuclease: *sbcD* (SYNPCC7002_A2342)

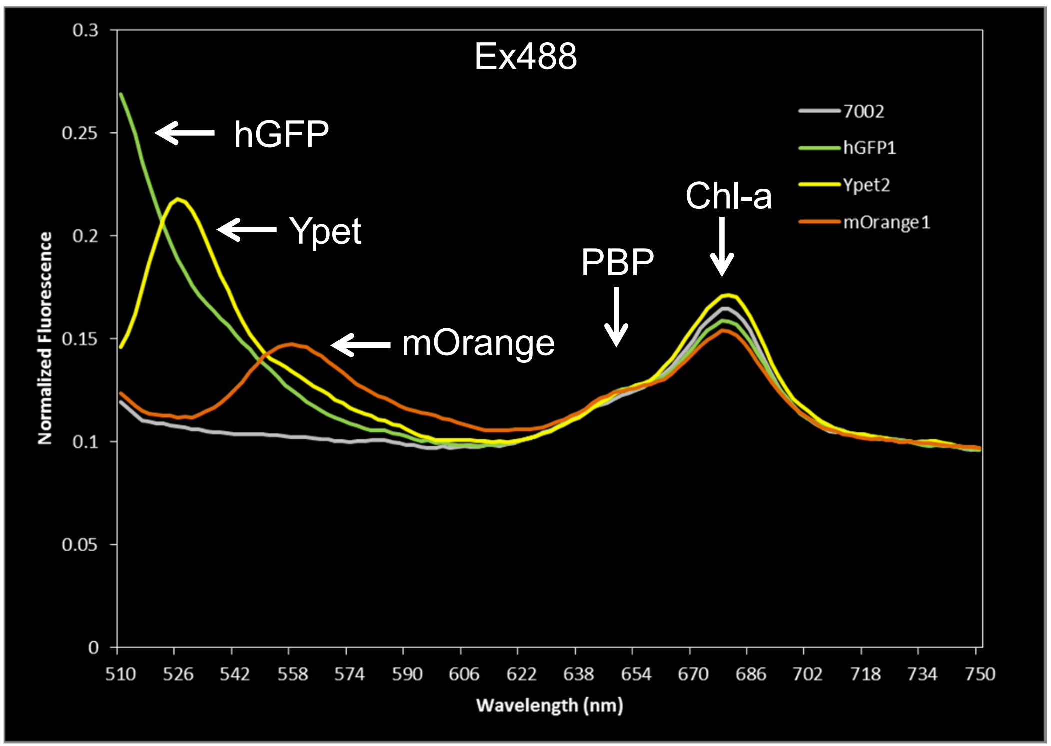


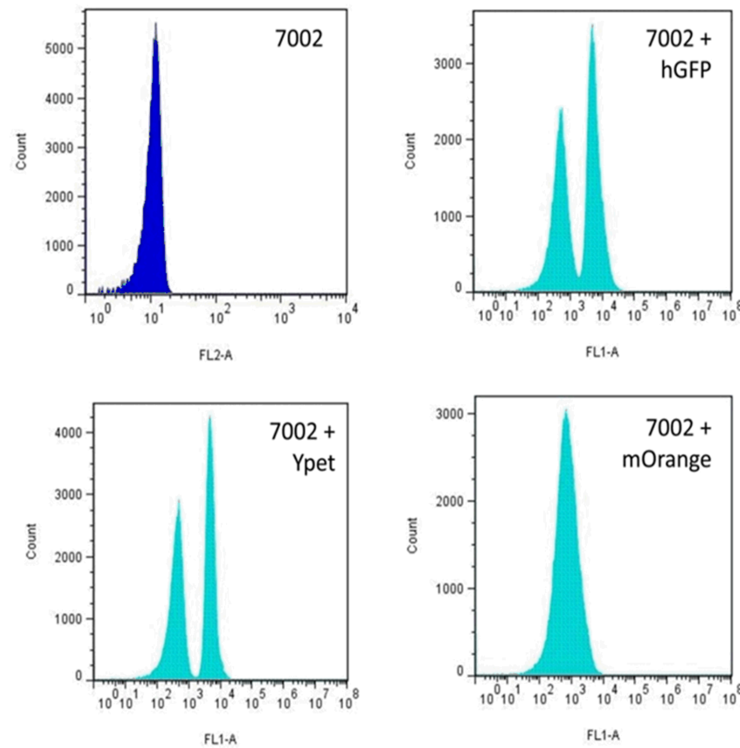


Reporters for *Synechococcus* sp. PCC 7002

- hGFP (Ex460-490, Em510)
- Ypet (Ex515, Em530)
- mOrange (Ex540, Em560)

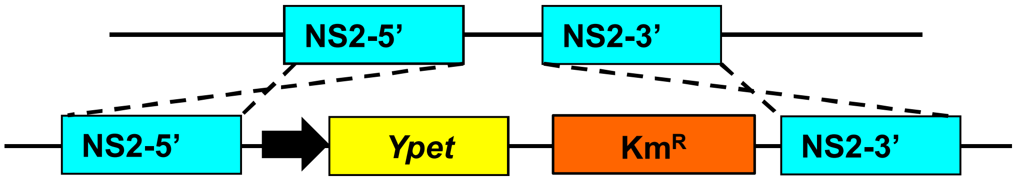






Synechococcus sp. PCC 7002 Promoters

- Selected 24 native promoters for characterization with Ypet fluorescence
- Selection based on previous RNA-seq results[7]
  - Expression levels: 10^{-2} – 10^{-5}
  - Regulatory patterns: constitutive, linear phase, and stationary phase
- Promoter: 500 bp upstream of orf



- Characterized Ypet expression under both continuous light and diurnal light conditions (~ 60 μmol/m^2s)

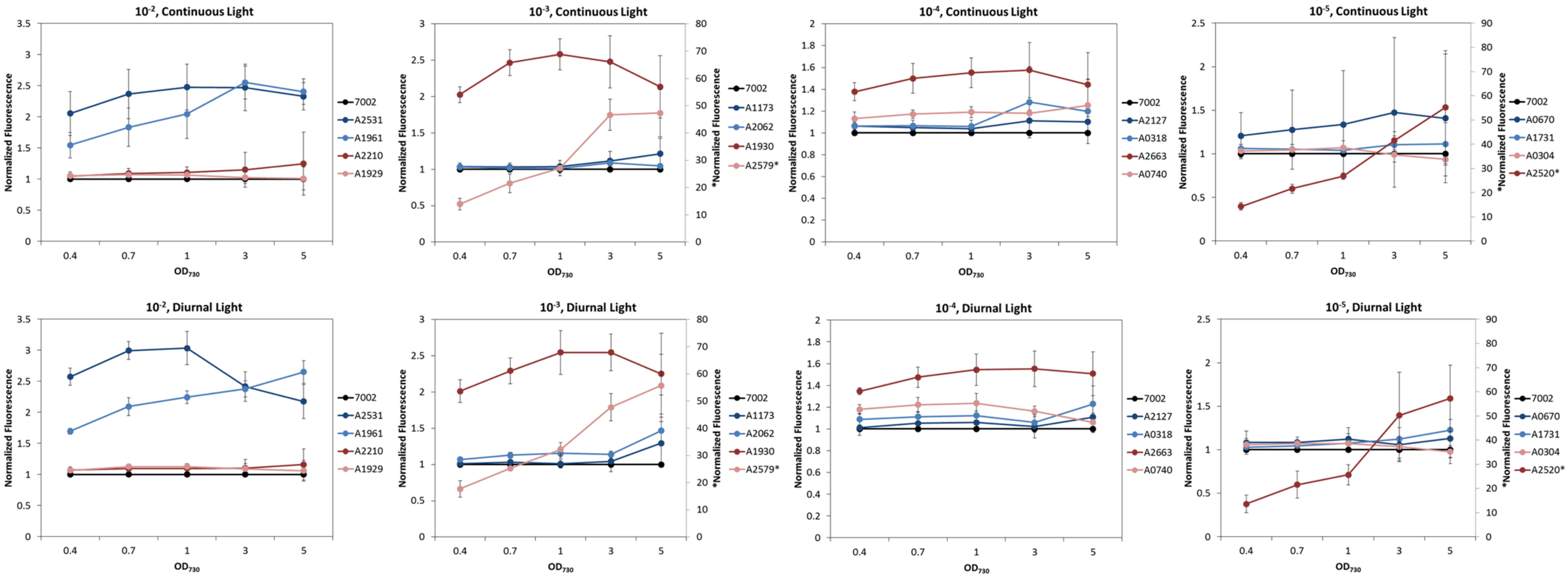
Locus Tag	Regulation	Expression Level	Gene Product
A0670	Constitutive	10^{-5}	conserved hypothetical protein
A1731	Constitutive	10^{-5}	hypothetical protein
A2127	Constitutive	10^{-4}	*accC*, acetyl-CoA carboxylase, biotin carboxylase
A0318	Constitutive	10^{-4}	outer membrane protein, OMP85 family, UDP-3-0-acyl N-acetylglucosamine deacetylase
A1173	Constitutive	10^{-3}	polyketide synthase
A2062	Constitutive	10^{-3}	*fusA*, ribosomal protein S10, translation elongation factor Tu
A2531	Constitutive	10^{-2}	conserved hypothetical protein
A1961	Constitutive	10^{-2}	*psaA*, photosystem I P700 chlorophyll A apoprotein A1
A2520	Linear phase	10^{-5}	conserved hypothetical membrane protein
A0304	Linear phase	10^{-5}	conserved hypothetical proteins
A2663	Linear phase	10^{-4}	*bfr*, bacterioferritin
A0740	Linear phase	10^{-4}	ATP synthase subunit I
A1930	Linear phase	10^{-3}	*apcA*, allophycocyanin α subunit
A2579	Linear phase	10^{-3}	hypothetical protein
A2210	Linear phase	10^{-2}	*cpcA*, phycocyanin α subunit
A1929	Linear phase	10^{-2}	*apcB*, allophycocyanin β subunit
A0255	Stationary phase	10^{-5}	glycosyl transferase, WecB/TagA/CpsF family
A2165	Stationary phase	10^{-5}	conserved hypothetical proteins
A2595	Stationary phase	10^{-4}	conserved hypothetical protein
A2596	Stationary phase	10^{-4}	conserved hypothetical protein
A0047	Stationary phase	10^{-3}	conserved hypothetical protein, CheW-like domain; methyl-accepting chemotaxis protein
A1181	Stationary phase	10^{-3}	ATPase, AAA family domain protein
A1962	Stationary phase	10^{-2}	*psaB*, photosystem I protein A2
A2813	Stationary phase	10^{-2}	S-layer like protein; probable porin

7. Ludwig M and Bryant DA. 2012. *Acclimation of the global transcriptome of the cyanobacterium Synechococcus sp. PCC 7002 to nutrient limitations and different nitrogen sources*. Front. Microbiol. 3: 145.

Sandia National Laboratories

Synechococcus sp. PCC 7002 Promoters

10^{-2} | 10^{-3} | 10^{-4} | 10^{-5}



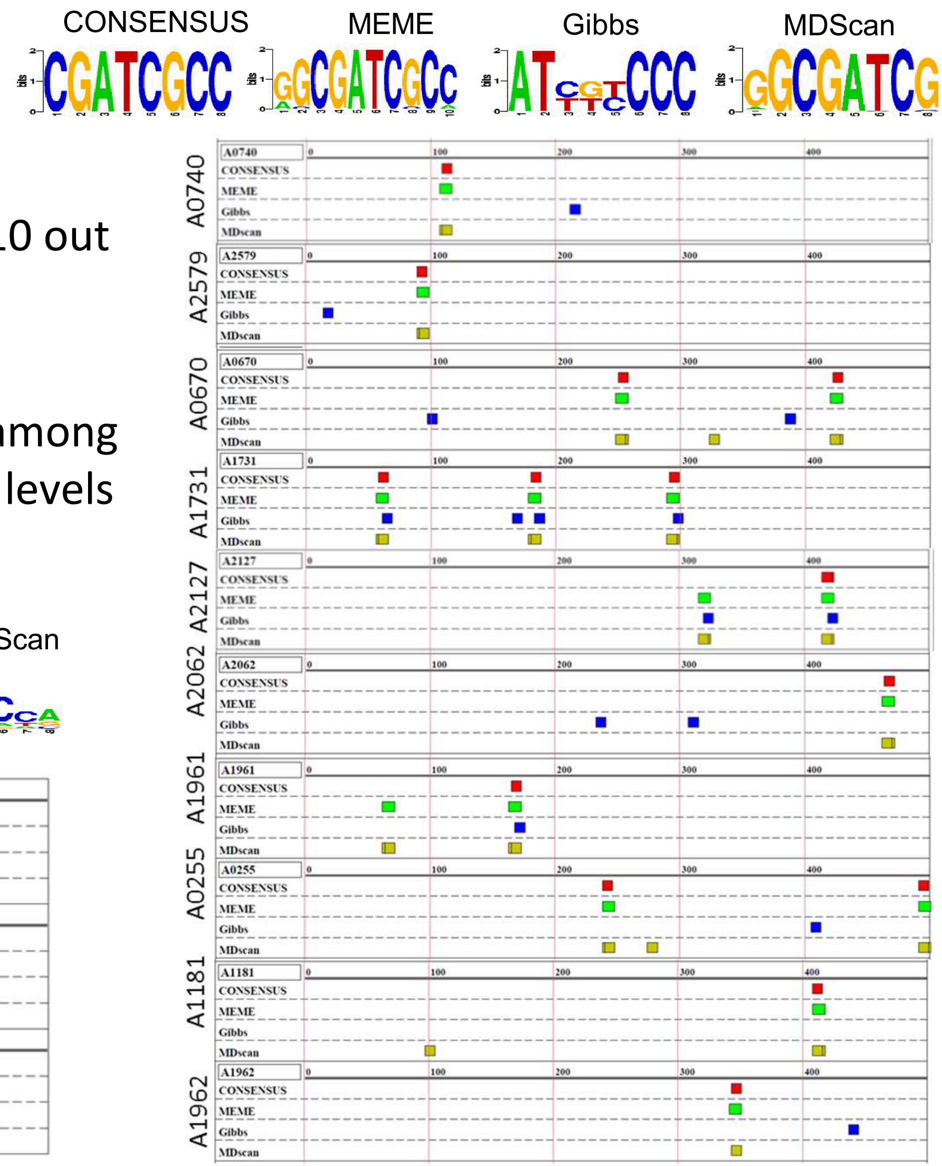
Constitutive
Linear Phase

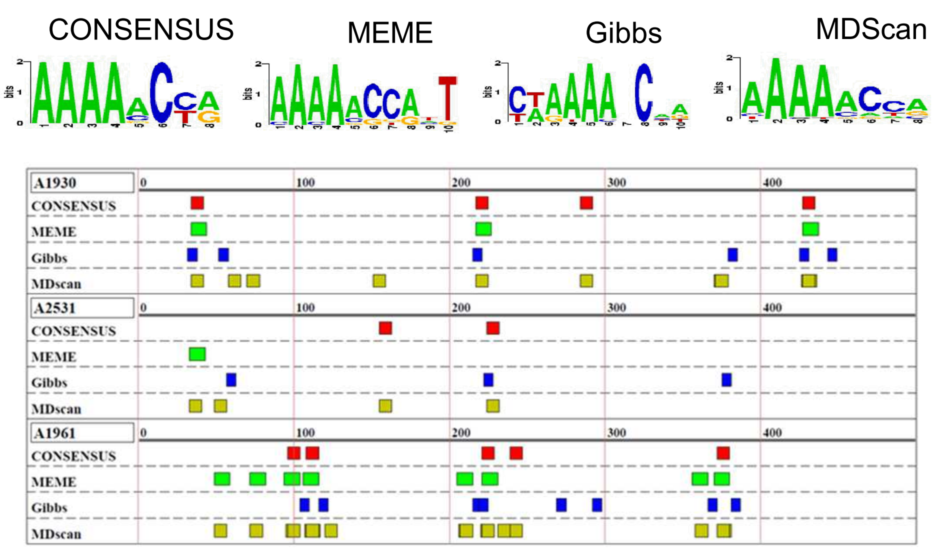
- Promoter strength does not always correlate with RNA-seq results
- Regulatory patterns of Ypet expression do not match RNA-seq results
- Dirunal light conditions do not affect the overall expression levels and regulatory patterns of promoters over 3 weeks of cultivation (may have daily, short time scale effects)
- Low dynamic range of expression for most 7002 promoters

Synechococcus sp. PCC 7002 Promoter Motifs

Sandia National Laboratories

- Promoter motif analysis with Melina II (4 motif finders: CONSENSUS, MEME, Gibbs, MDScan)
- Promoter motif GGCGATCG identified in 10 out of 30 promoter regions
  - No observable expression trend
- Promoter motif AAAAACCA is consistent among promoters showing moderate expression levels (2- to 3-fold higher).





Conclusions

- Advancements in engineering cyanobacteria for FFA production
  - Successful FFA production and excretion in two cyanobacterial hosts
  - Investigation of inducible and native promoters for gene expression
- Biofuel toxicity is a limiting factor for cyanobacterial fuel production
  - Physiological effects: cell growth, stress, cell death, photosynthetic yield, photosynthetic pigments
  - Identification of target genes affecting cell physiology during FFA production (RNA-seq, mutants)
- Development of a cyanobacterial chassis will enable optimization of cyanobacterial fuel production
  - Developed tools (reporters and promoters) and optimized transformation for *Synechococcus* sp. PCC 7002 as a cyanobacterial chassis

Acknowledgements

Staff

Howland D.T. Jones (HyperImage Solutions)

Omar F. Garcia

Undergraduate Interns

Christine Trahan

Travis Jensen

Lucas Strickland

Michelle Raymer

Managers

Anthony Martino

Eric Ackerman

Jim Carney

Lipid analysis

Kansas Lipidomics Research Center (KLRC)
Kansas State University

Sequencing (RNA-Seq)

Los Alamos National Laboratory (LANL)

Funding

President Harry S. Truman Fellowship in National Security Science and Engineering

Early Career LDRD Project