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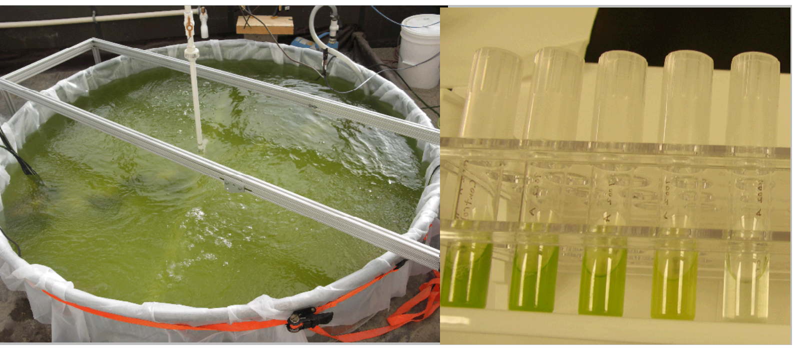

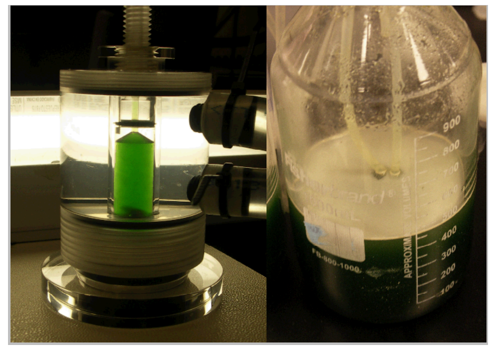

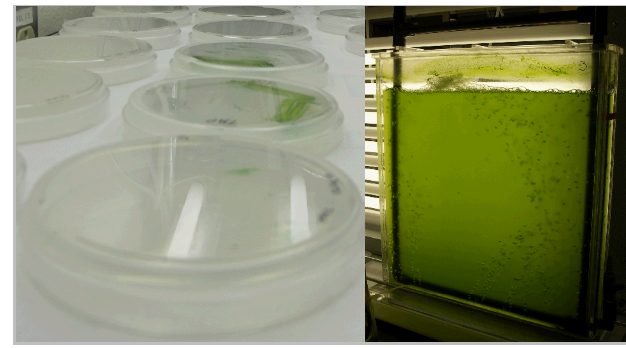

Blue-Green Biofuels: Engineering Cyanobacterial Free Fatty Acid Production

Anne M. Ruffing

PNNL Biosciences Seminar

April 23, 2013

U.S. DEPARTMENT OF ENERGY

NNSA National Nuclear Security Administration

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Outline

- Background on Cyanobacterial Biofuels
- Project Objectives:
    - Engineering *Synechococcus elongatus* PCC 7942 for FFA Production
    - Characterizing the Physiological Effects of Cyanobacterial FFA Production
    - Seq-ing Targets for Improved Physiology and FFA Productivity
    - *Synechococcus* sp. PCC 7002 as host for FFA Production
    - Biofuel Toxicity and Cyanobacterial Tolerance
- Conclusions and Future Work

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.

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

Why Cyanobacteria?

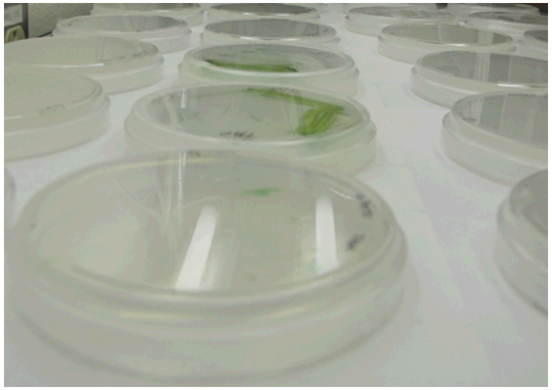
Advantages of Cyanobacteria for Fuel Production

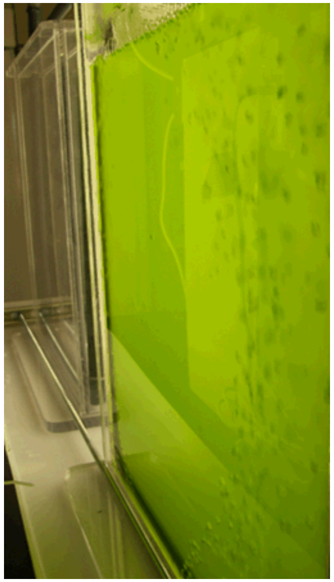
Desirable strain traits

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

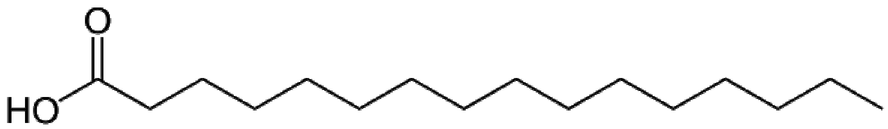




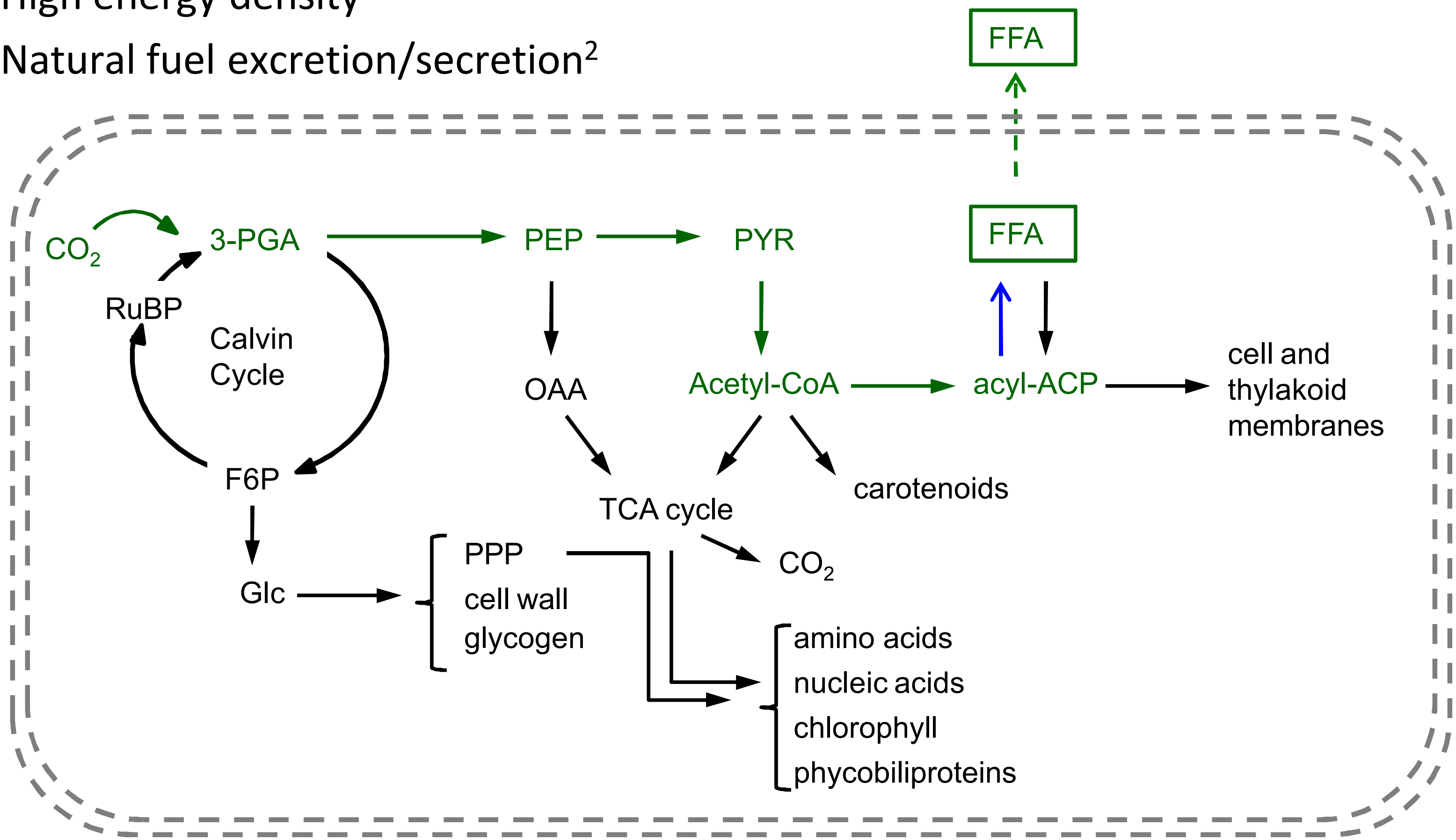
Target Fuel: Free Fatty Acids (FFA)

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.

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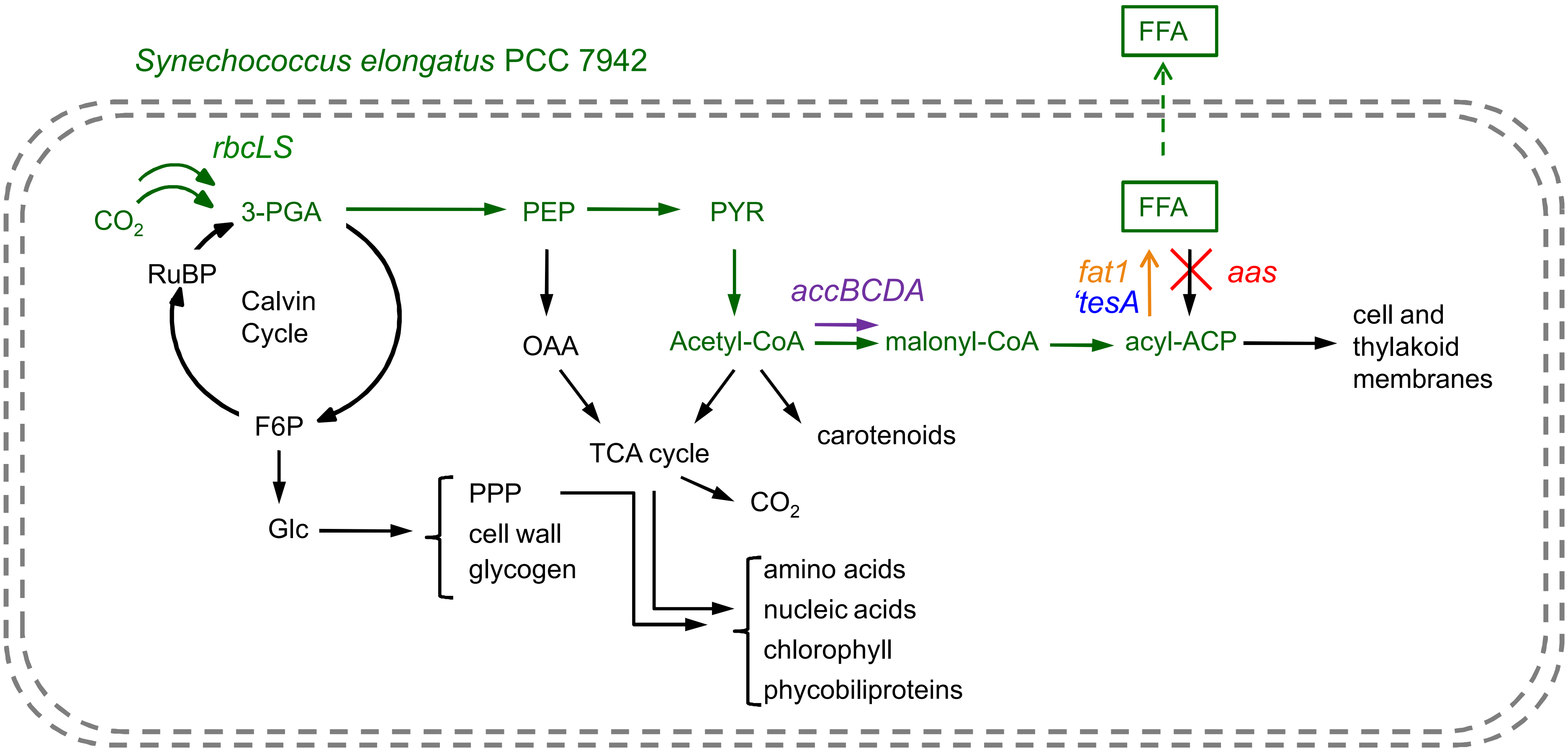
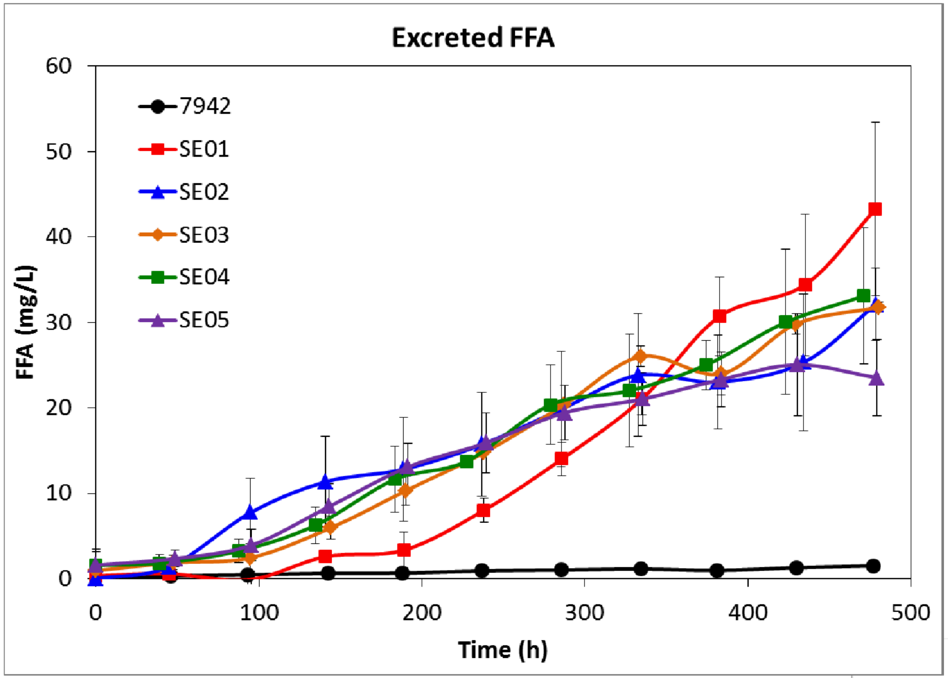
Genetic Engineering of Cyanobacteria to Produce FFA



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*

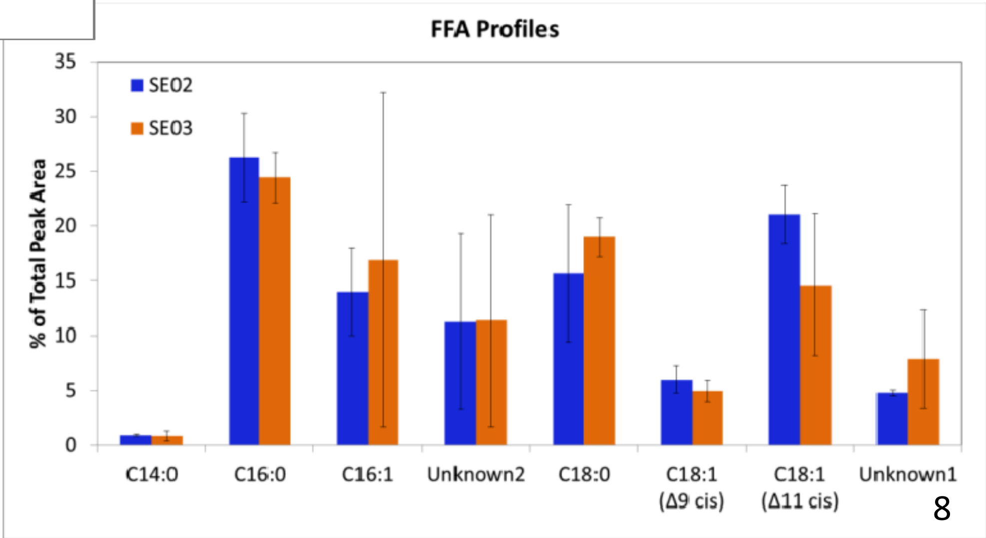
- *aas* – acyl-ACP synthetase / long-chain-fatty-acid CoA ligase
- *'tesA* – truncated thioesterase from *Escherichia coli*
- *fat1* – acyl-ACP thioesterase from *Chlamydomonas reinhardtii*
- *rbcLS* – native RuBisCO
- *accBCDA* – multi-subunit acetyl-CoA carboxylase from *C. reinhardtii* (chloroplast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



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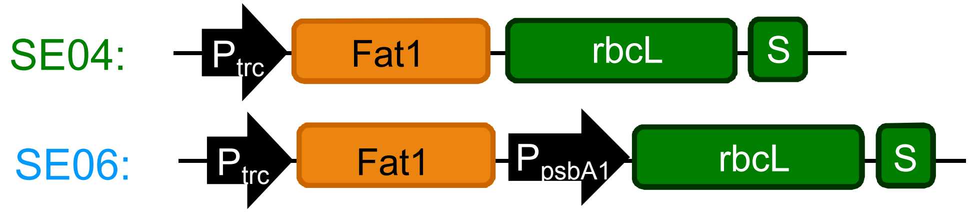
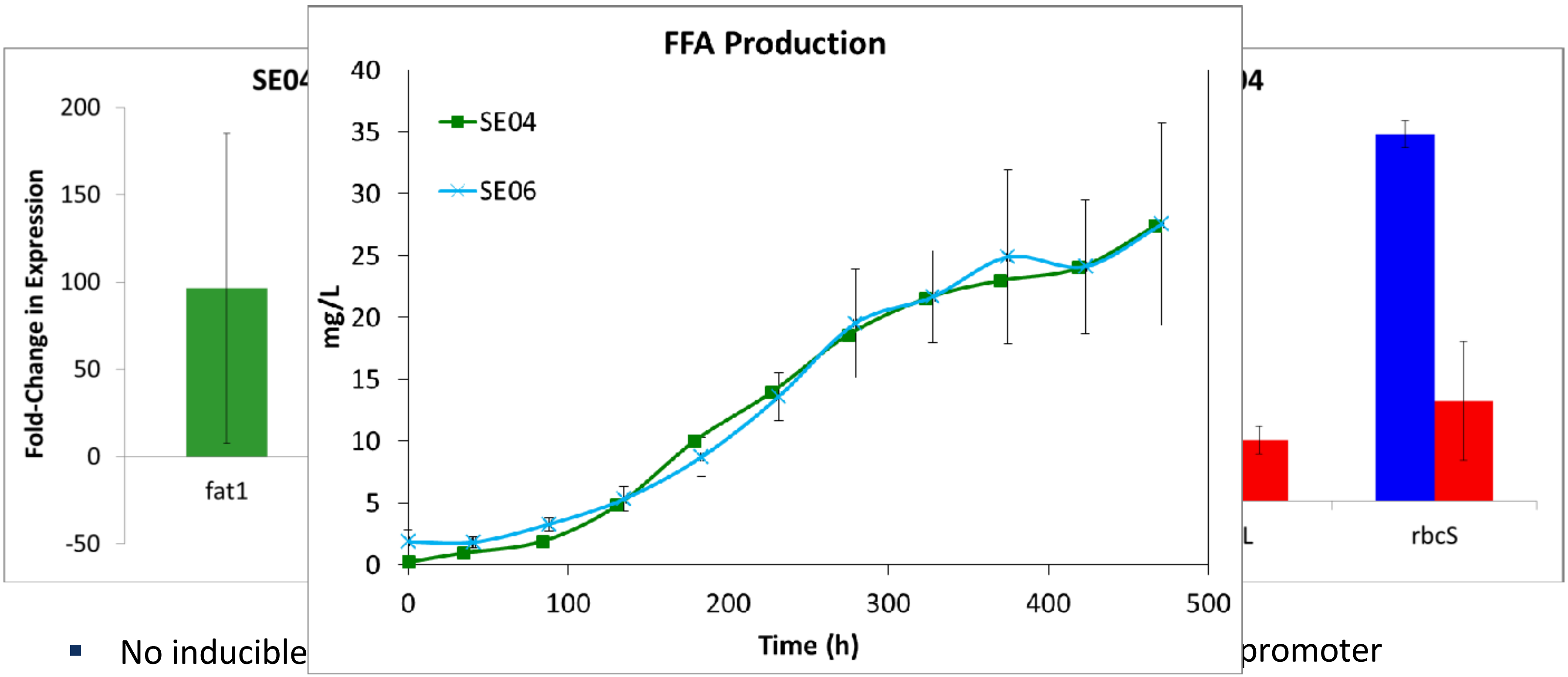
Does Increasing Gene Expression Improve FFA Production?

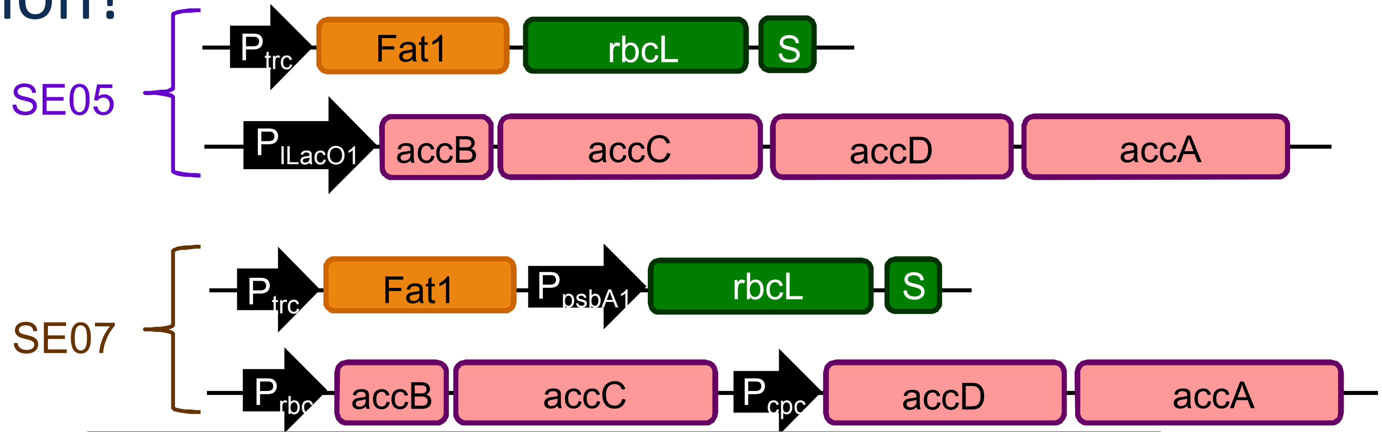


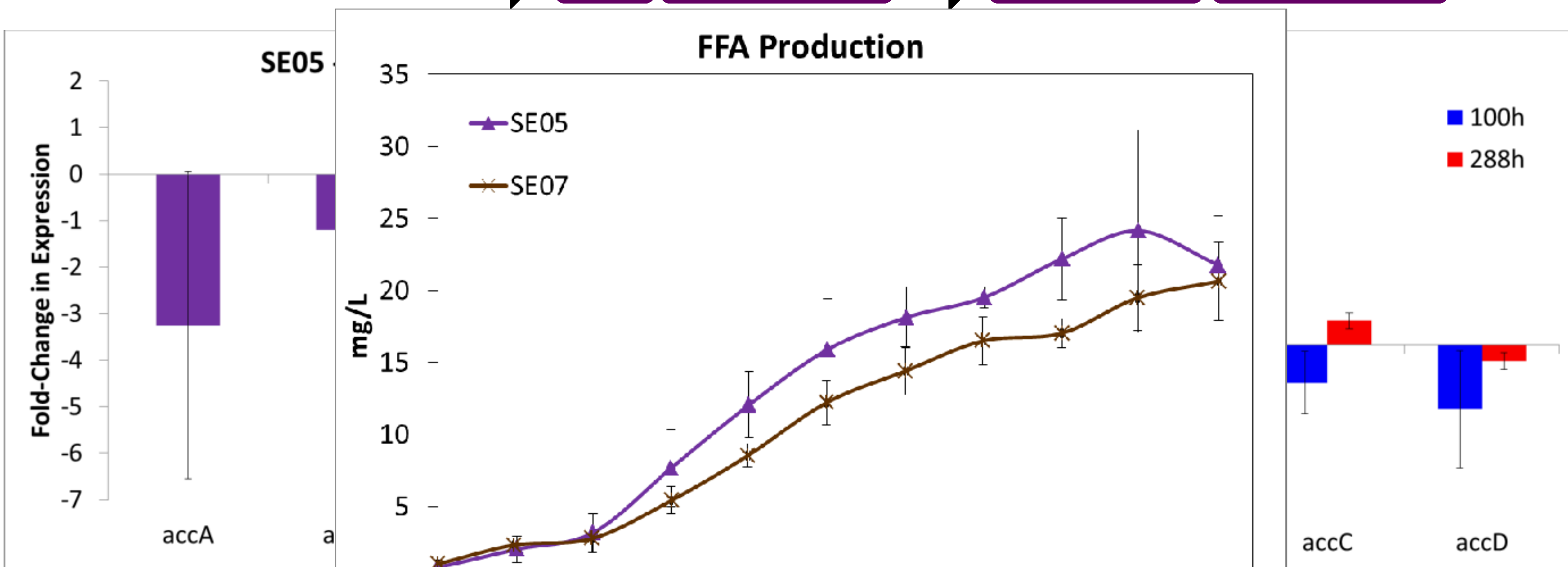


- No inducible

promoter improves *rbcLS* expression

Does Increasing Gene Expression Improve FFA Production?





- No inducible exp PLlacO1
- does not DA expression

Ruffing AM. Borrowing Genes from *Chlamydomonas reinhardtii* for Free Fatty Acid Production in Engineered Cyanobacteria. (2013) J. Appl. Phycol. Available Online.

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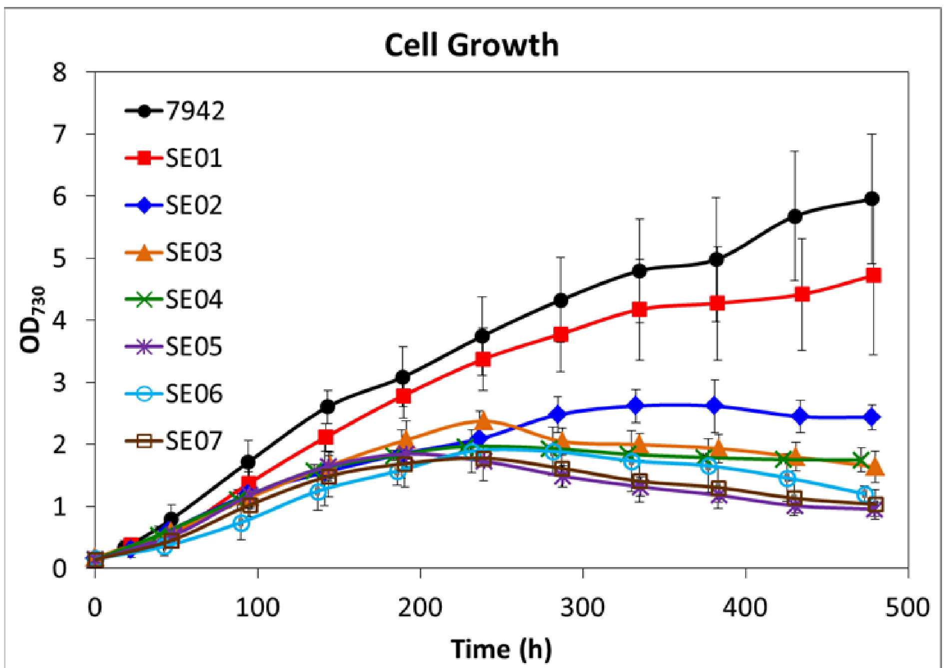
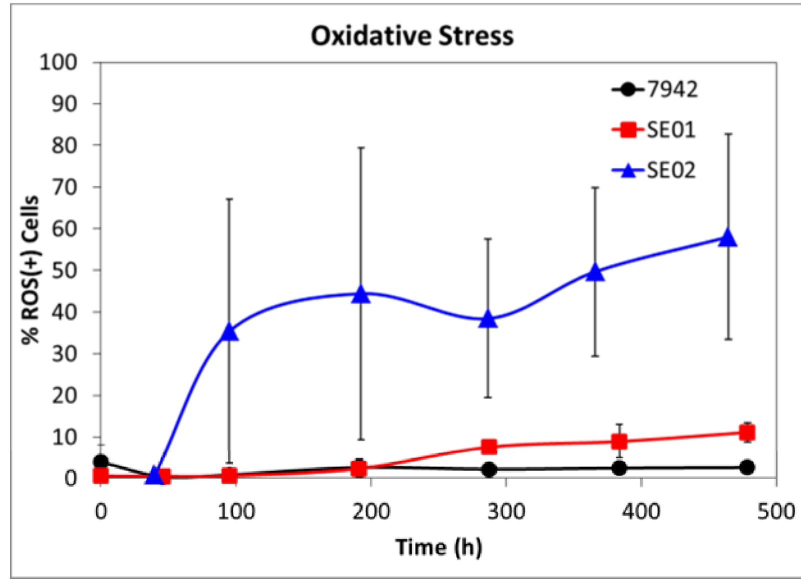
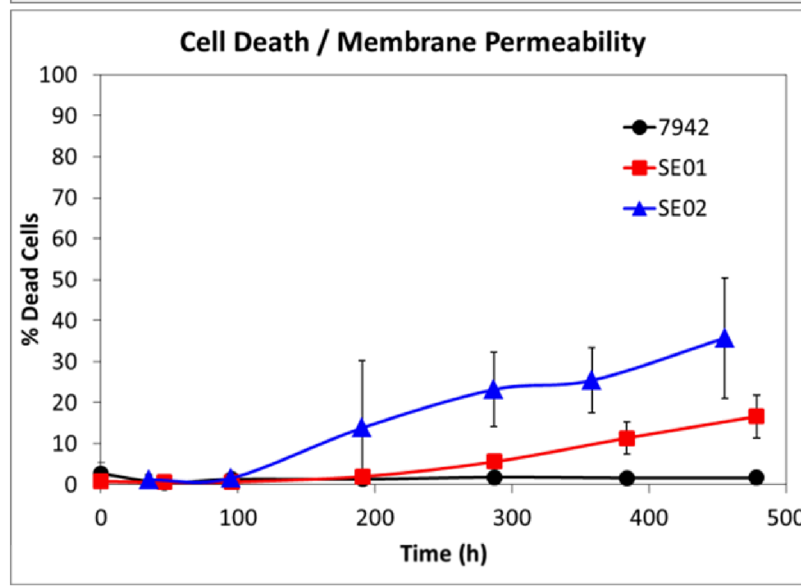
Physiological Effects: Growth, Stress, and Cell Death

- 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







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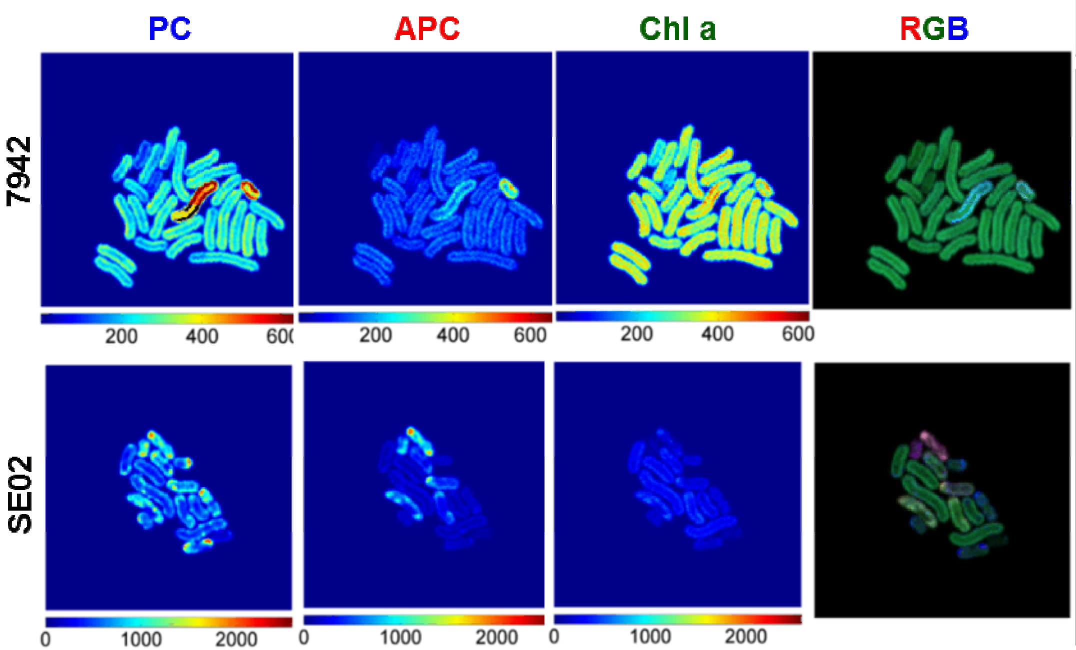
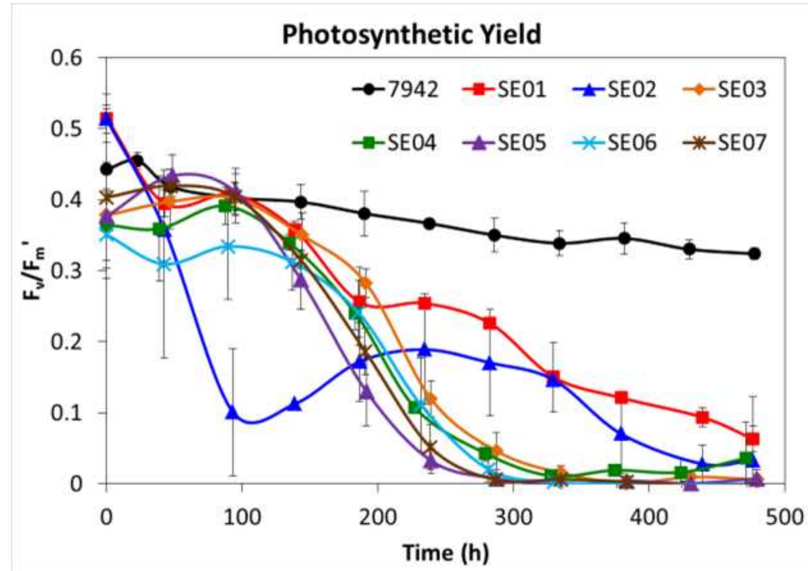
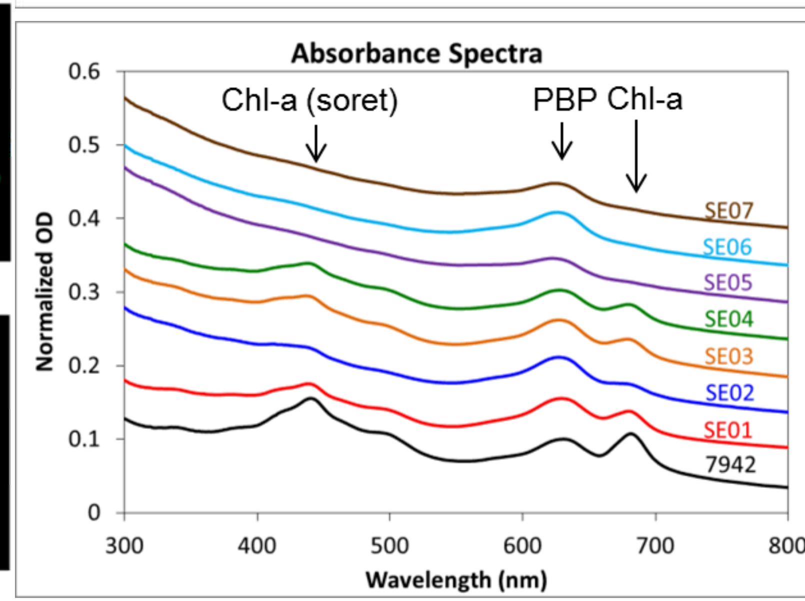
Photosynthetic Effects

- 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







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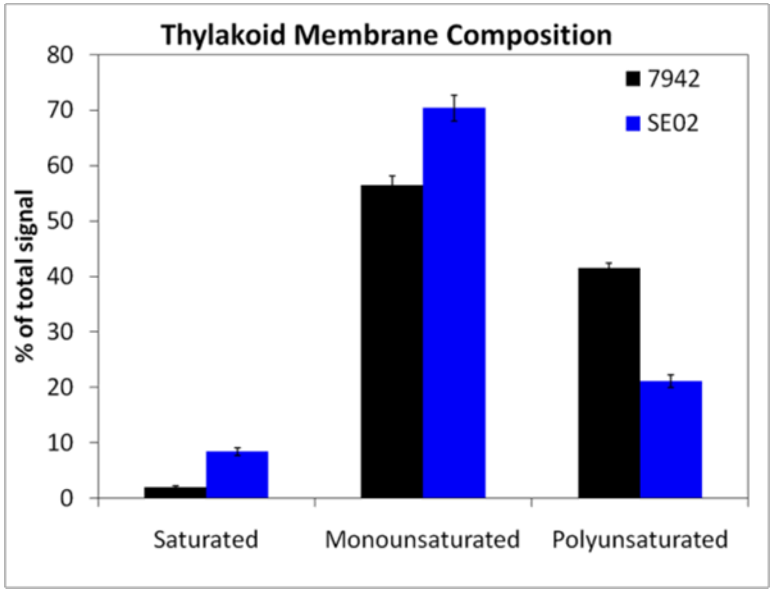
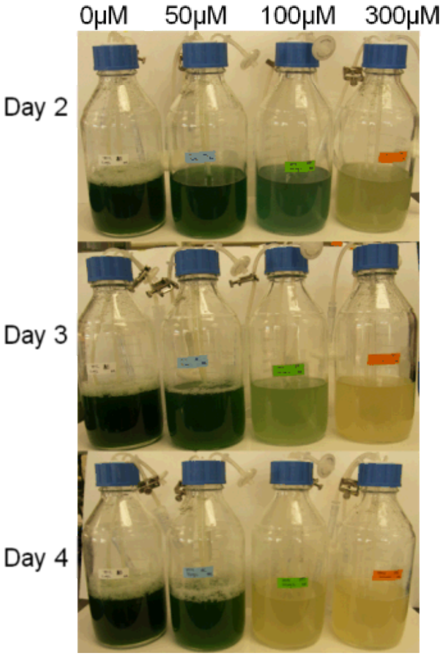
Possible Mechanisms of FFA Effects

Mechanism 1: Engineered strains have altered membrane composition

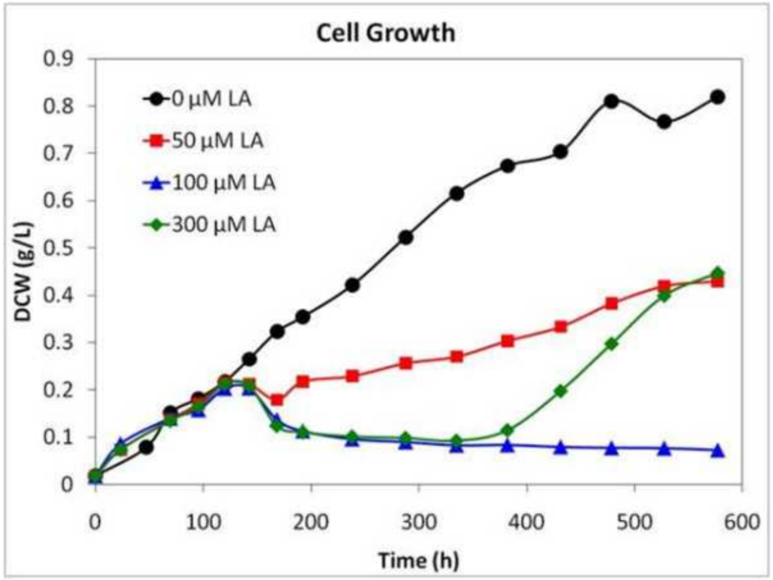
- Increased levels of saturated FA and lower levels of polyunsaturated FA in thylakoid membranes
- Leads to increased membrane viscosity and potential effect on phycobilisome attachment





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) Biotech. Bioeng. 109 (9): 2190-9.

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

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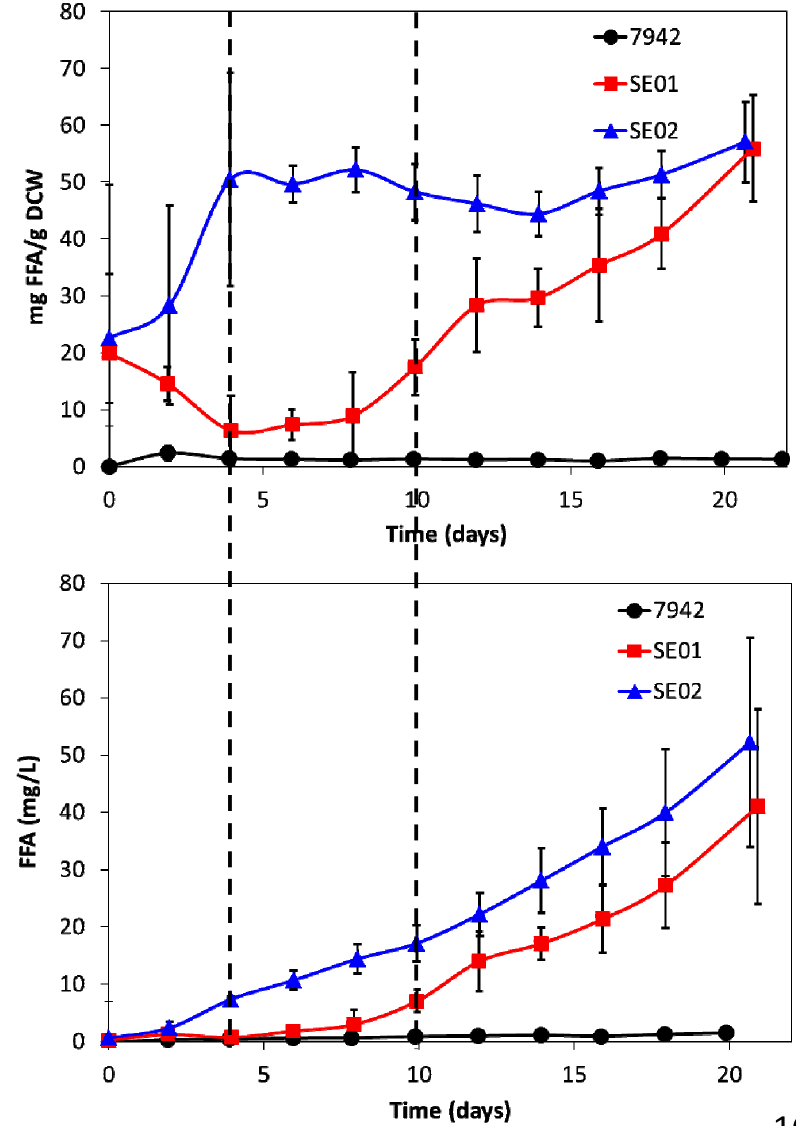
RNA-seq to identify genetic response to FFA production

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

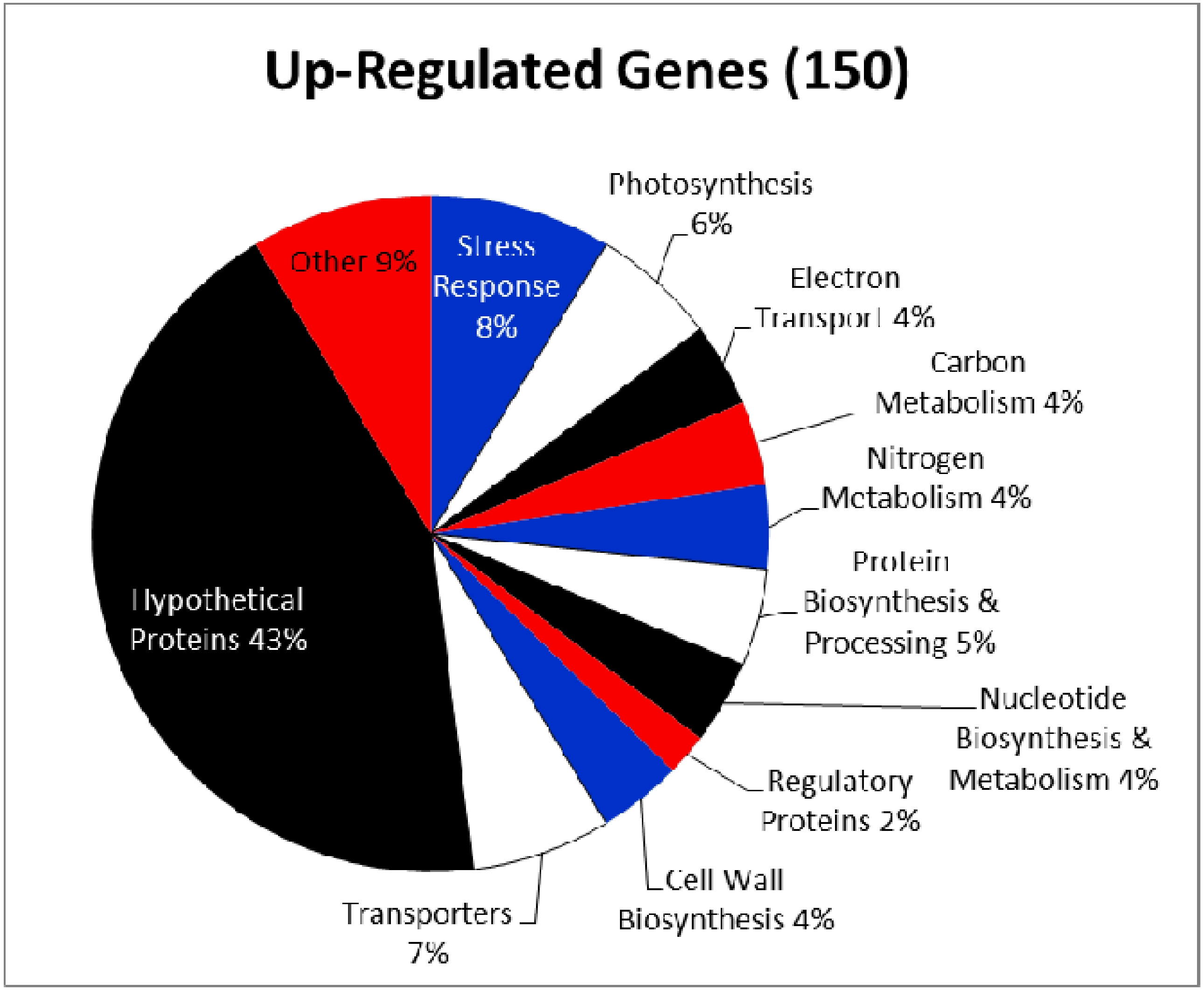
Differential gene expression comparisons:

	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.

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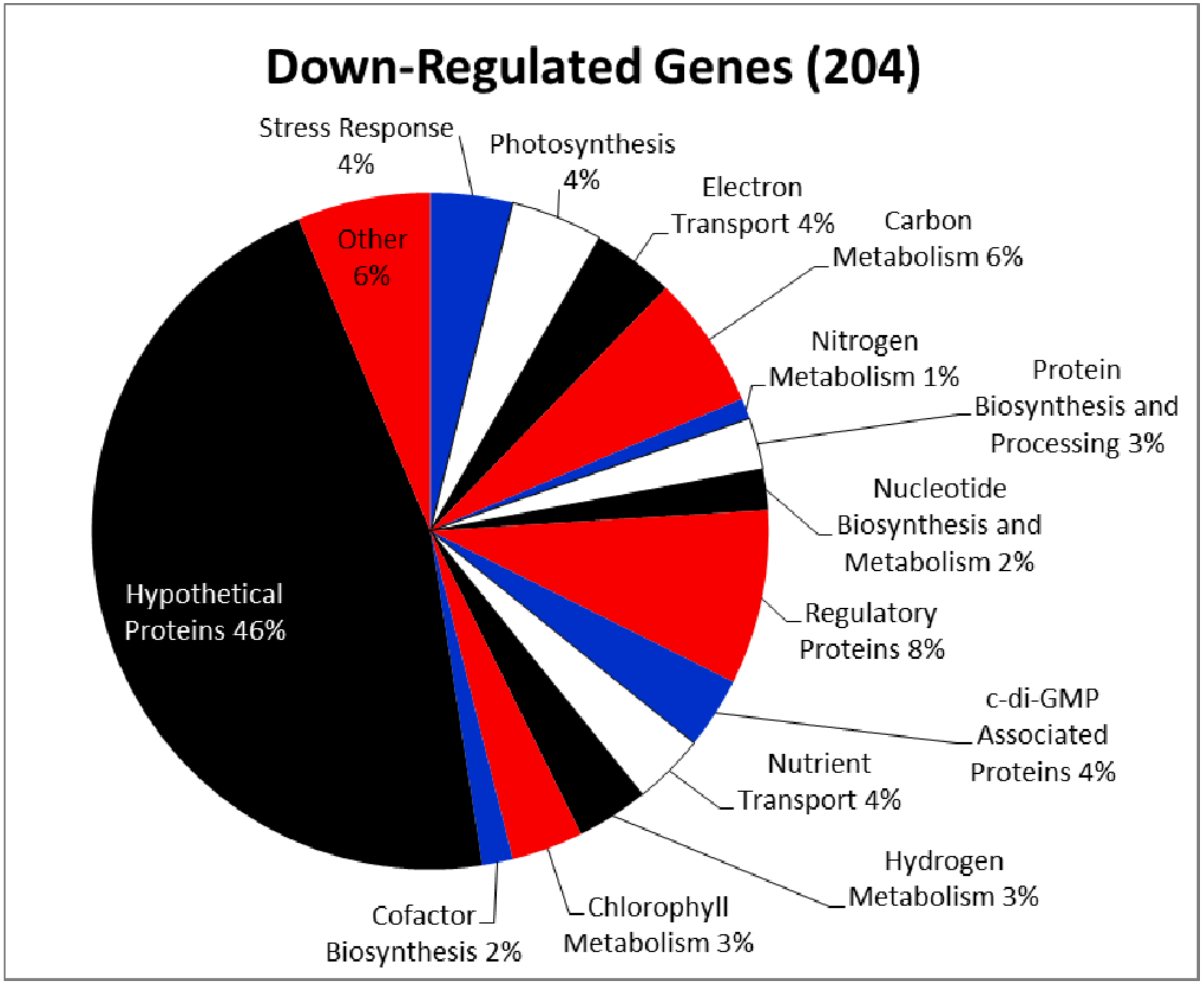


Differential Gene Expression: Fold change < -2, p-value < 0.05.

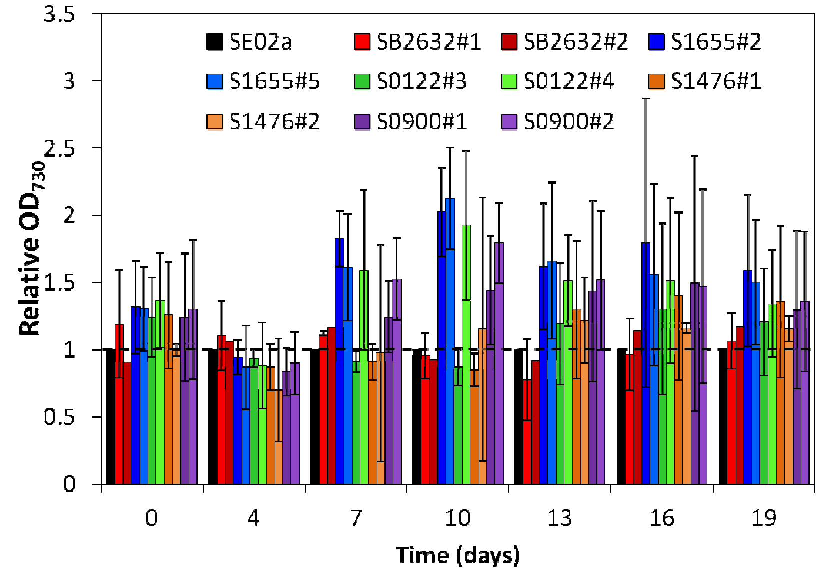
Identifying Targets for Improved FFA Production

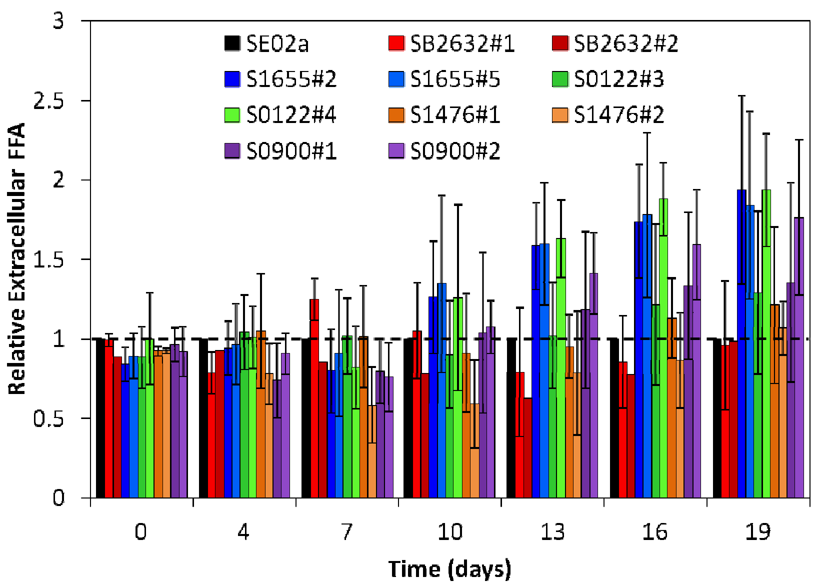
Locus	Product	Average FC	Targeted Mutagenesis
Hypothetical Proteins			
Synpcc7942_0444	hypothetical protein	3.27	Knockout
Synpcc7942_1561	hypothetical protein	2.67	Knockout
Synpcc7942_1023	hypothetical protein	2.15	Knockout
Synpcc7942_1476	hypothetical protein	-4.59	Overexpression
Synpcc7942_B2645	hypothetical 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

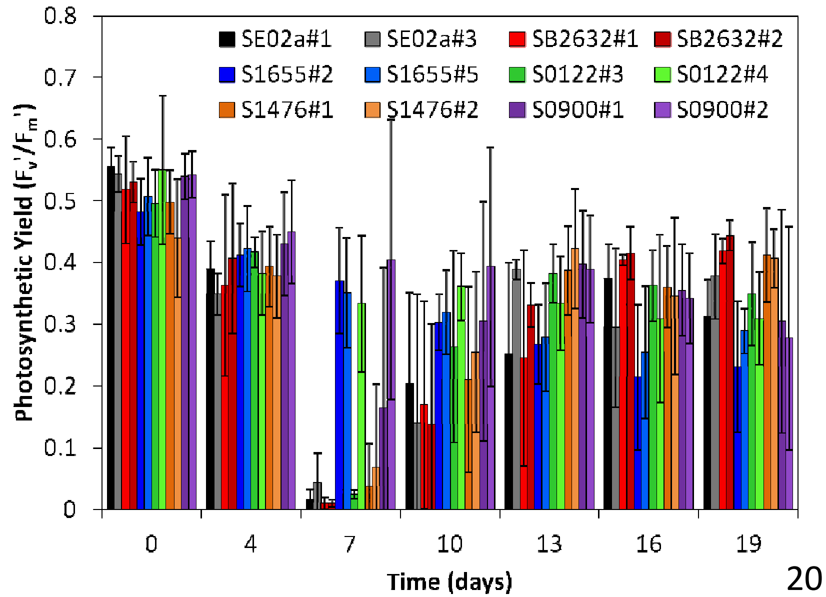
Hypothetical Protein Overexpression Mutants

Improved cell growth, photosynthetic yield, and FFA production:

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



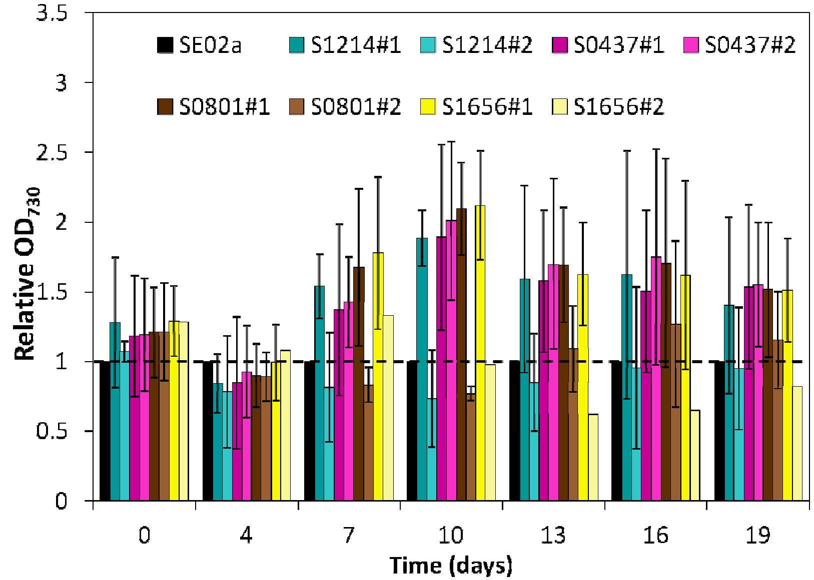


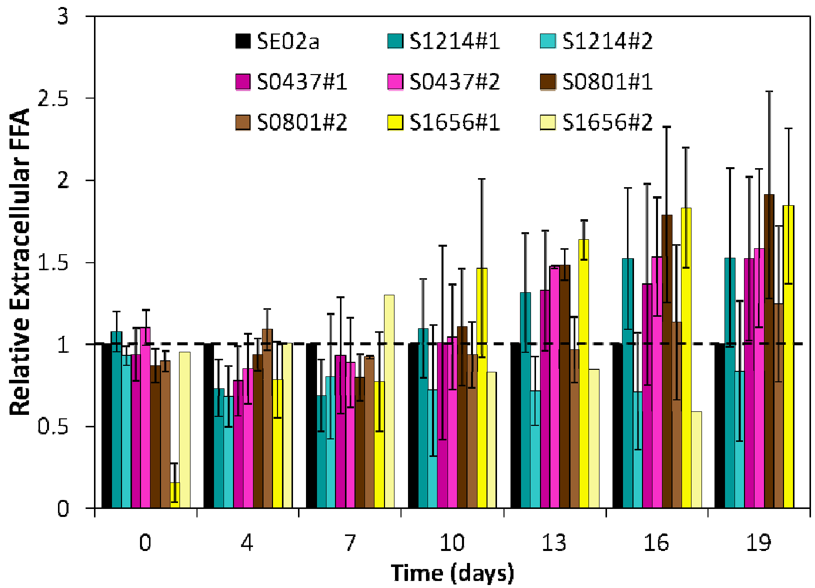


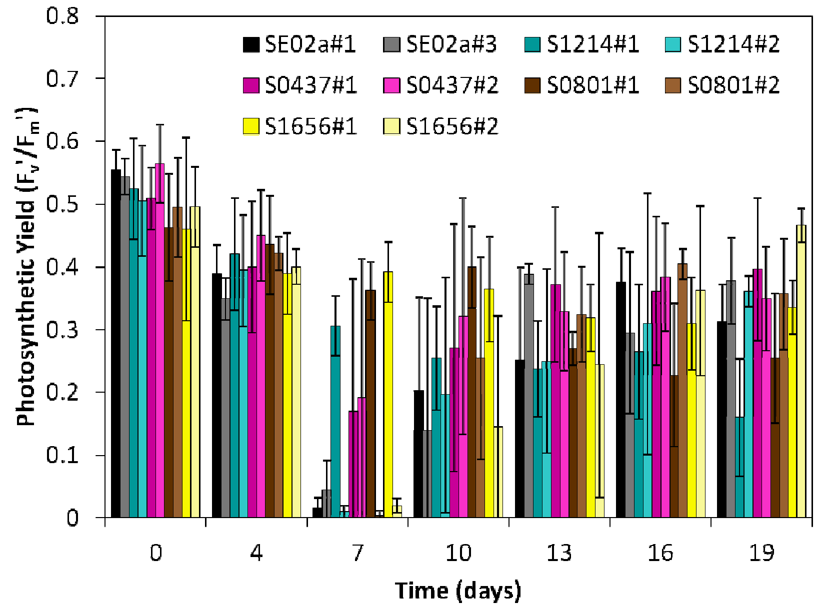
ROS-Degrading Protein Overexpression Mutants

Improved cell growth, photosynthetic yield, and FFA production:

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



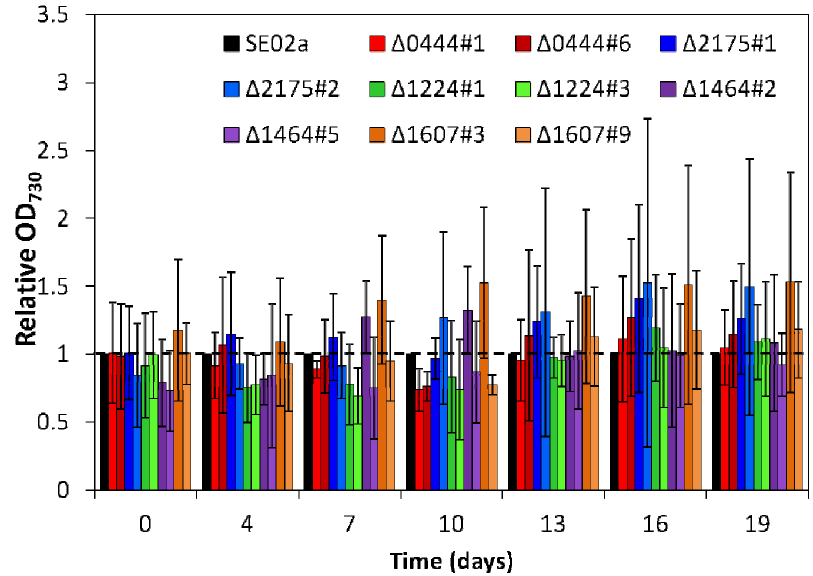


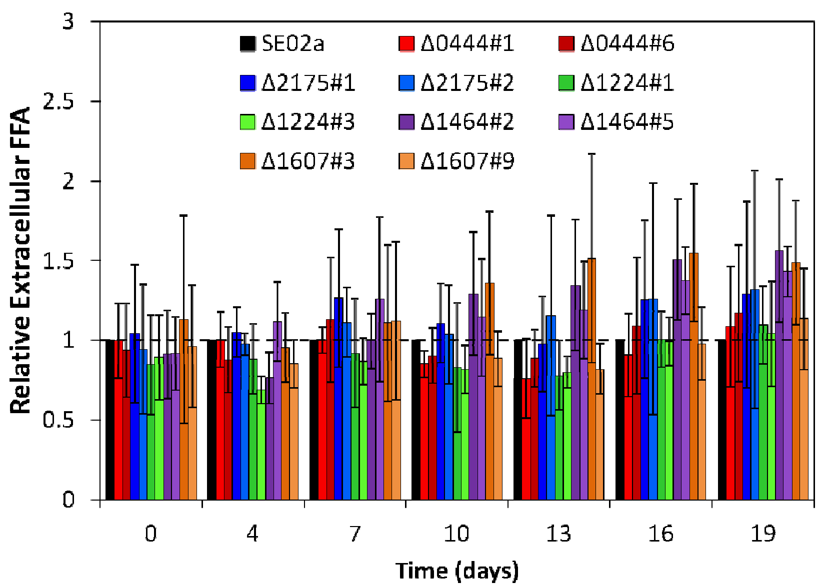


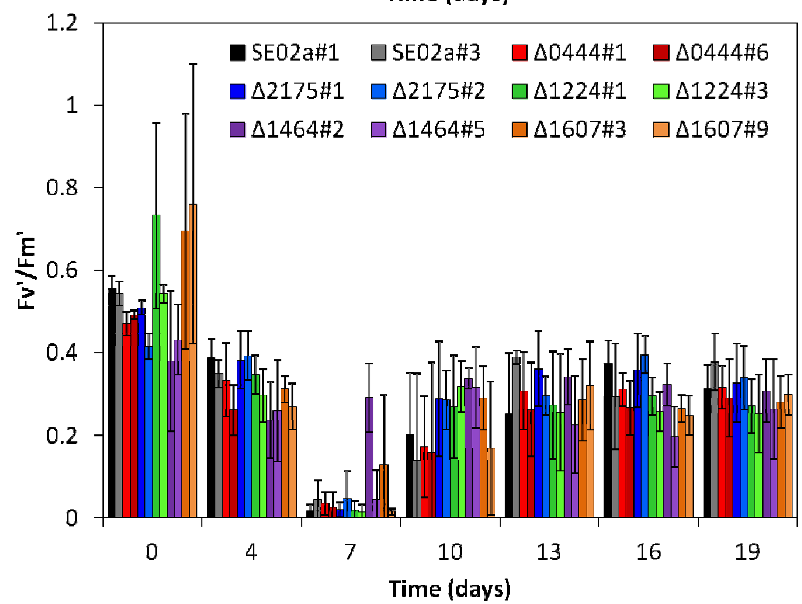
Knockout Mutants (Candidate FFA Export Proteins)

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

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







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Synechococcus sp. PCC 7002 as Host for FFA Production

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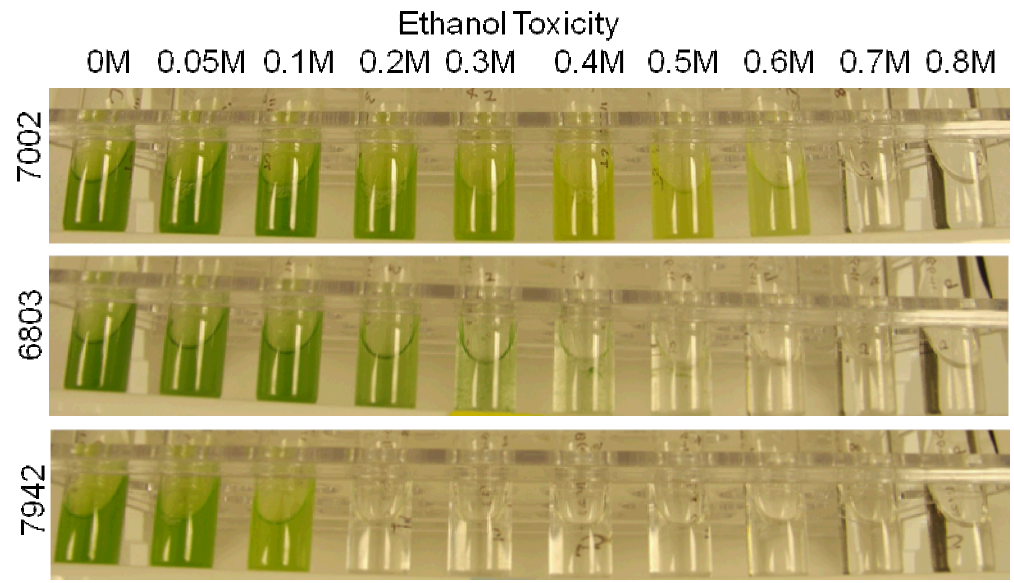
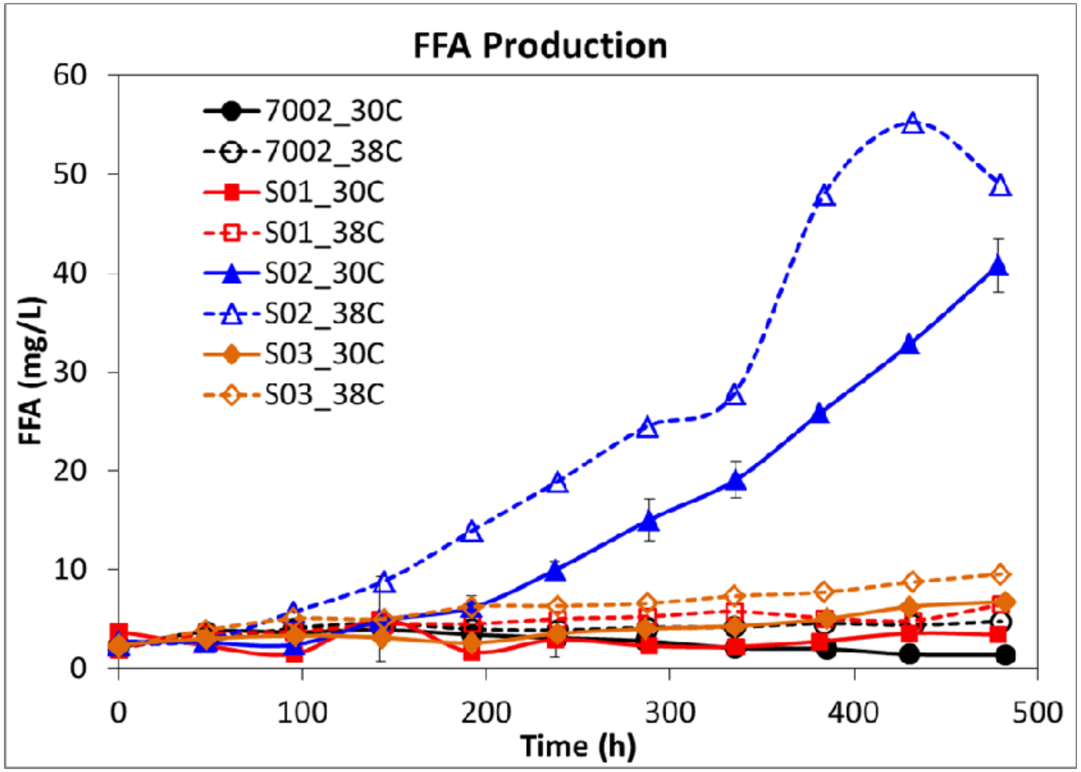
- Model cyanobacterium
  - Genetic tools available
  - Genome sequence
- Salt tolerance
- High light tolerance
- Biofuel tolerance



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	

FFA Production in 7002 Strains



7002: wild type

S01: Δ*fadD*

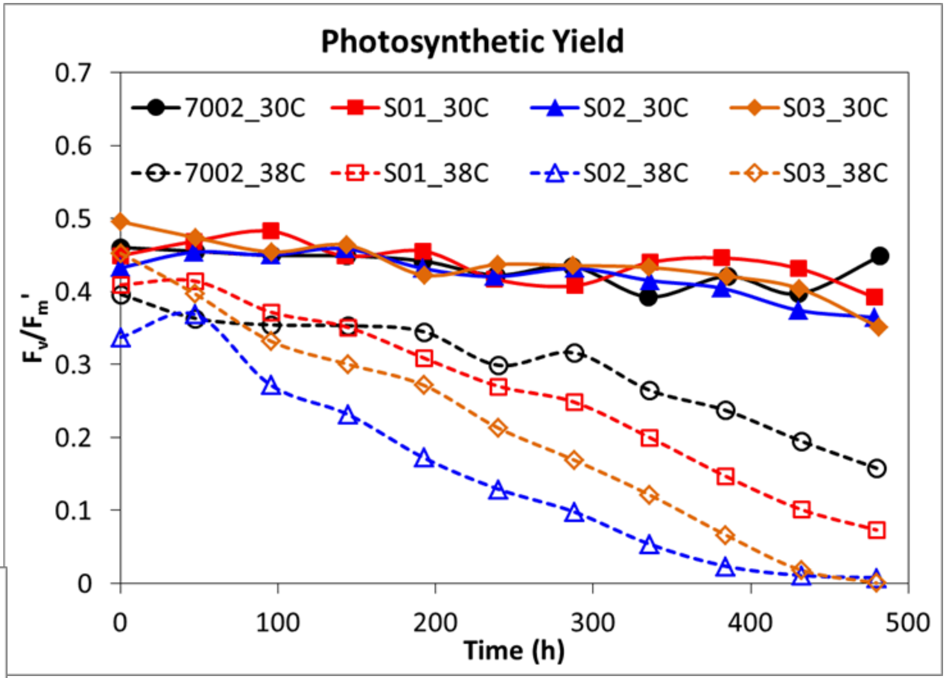
S02: Δ*fadD, 'tesA*

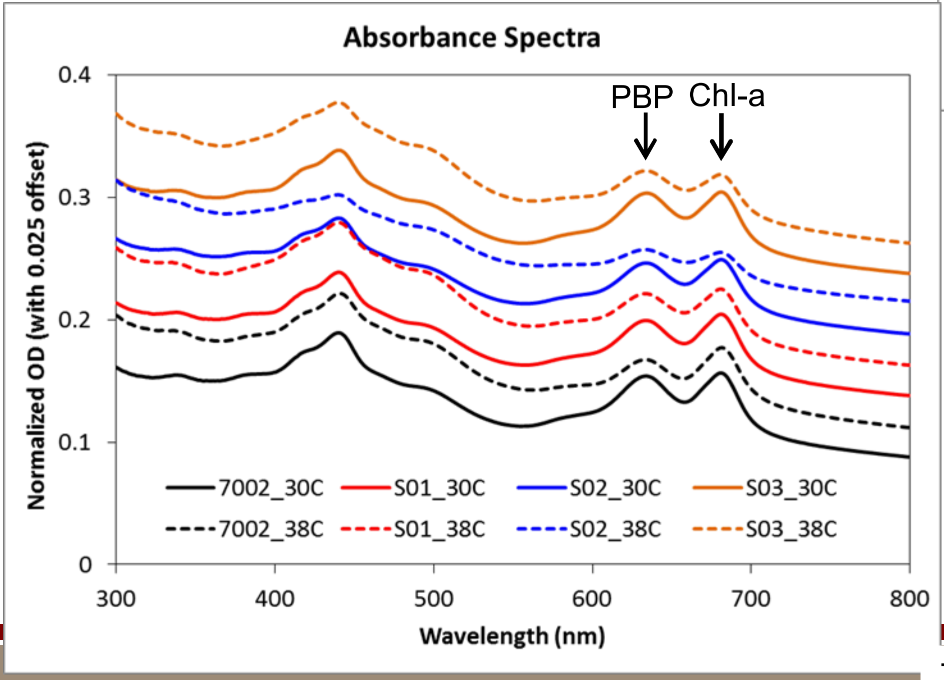
S03: Δ*fadD, fat1*

- 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.



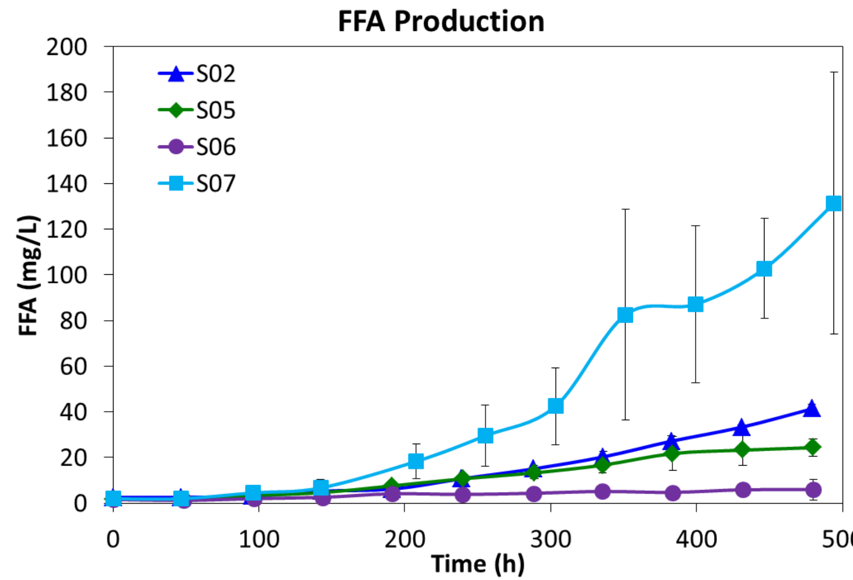


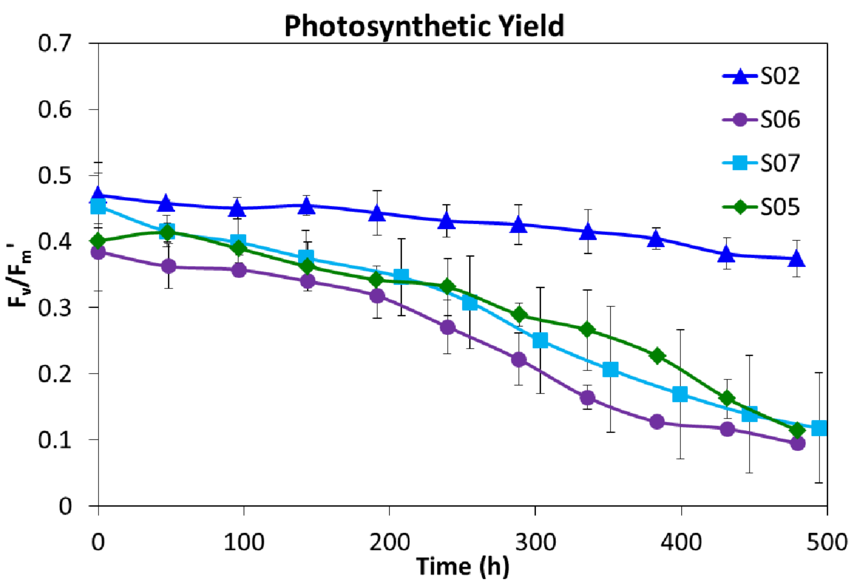
- No change in photosynthetic pigments for 7002 strains at 30°C.
- S02 shows degradation of both phycobiliprotein and Chl-a pigments at 38°C.
- This response differs from that of 7942, which showed selective degradation of Chl-a.

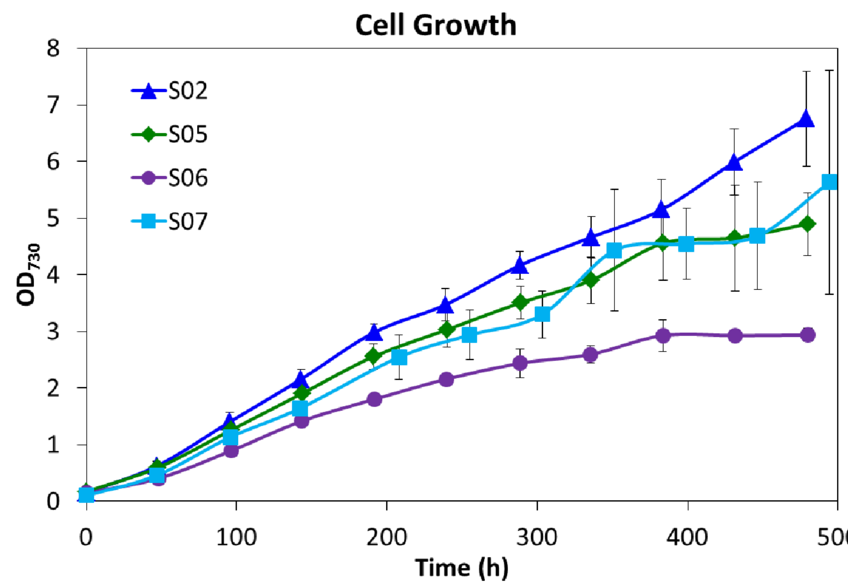
7002: wild type; S01: Δ*fadD;* S02: Δ*fadD, 'tesA;* S03: Δ*fadD, fat1*

RuBisCO Overexpression Improves FFA Yield

- 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*

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- Conclusions and Future Work

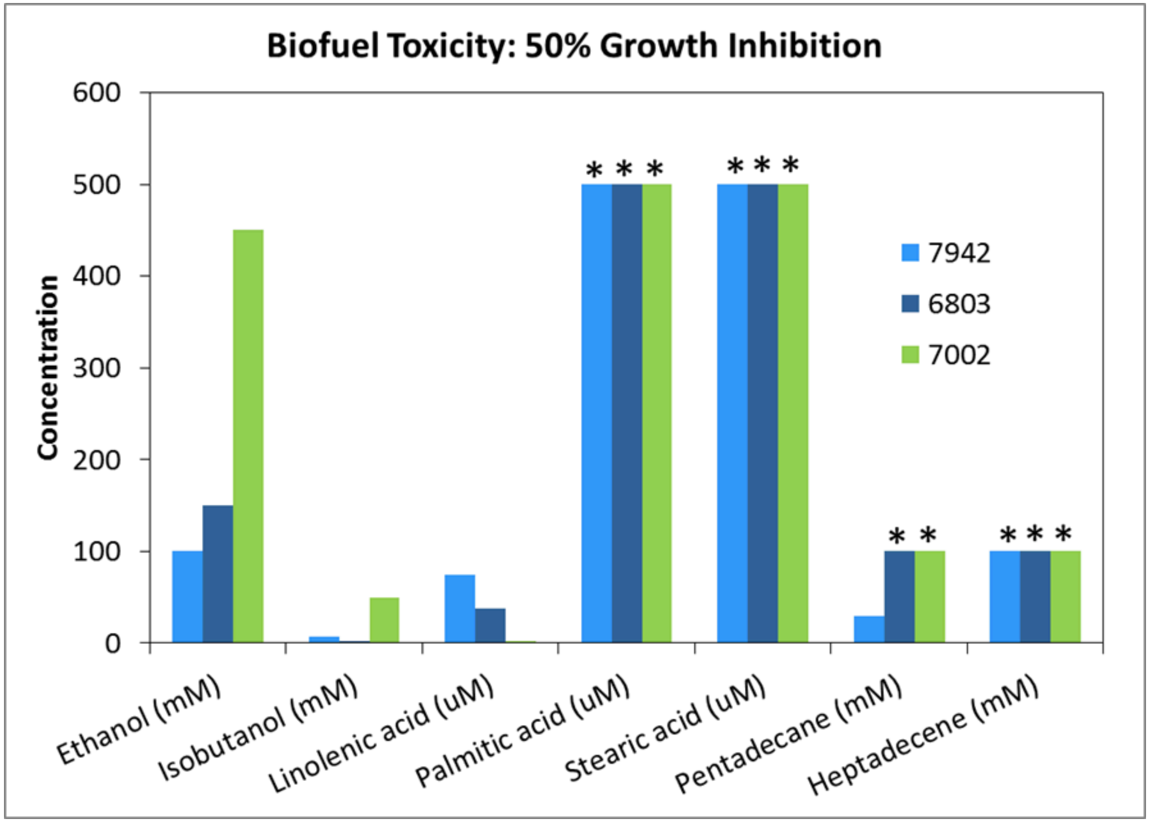
Biofuel Toxicity for Cyanobacteria

Model cyanobacteria:

- *S. elongatus* PCC 7942 (freshwater)
- *Synechocystis* sp. PCC 6803 (freshwater)
- *Synechococcus* sp. PCC 7002 (marine)

Biofuels:

- Short and long chain alcohols
- Fatty acids (saturated and unsaturated)
- Alkanes and alkenes



- 7002 has higher tolerance of short-chain alcohols
- 7942 has higher tolerance of unsaturated fatty acids
- Saturated fatty acids and alkanes/alkenes do not appear to be toxic to cyanobacteria

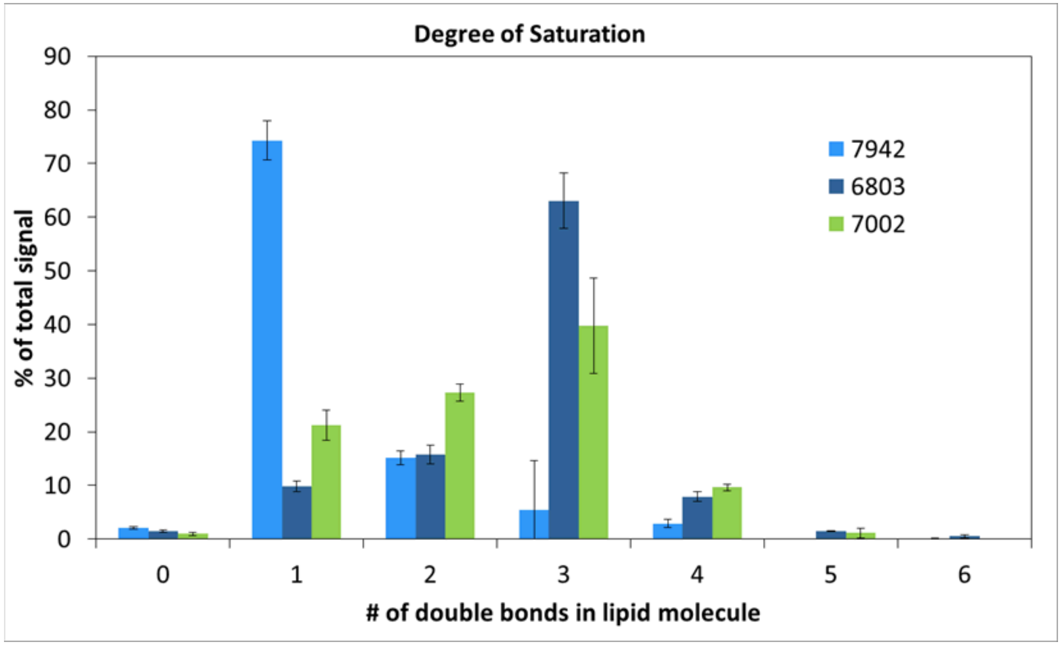
Mechanisms of Biofuel Tolerance: Membrane Structure/Permeability

ESI/MS analysis of membranes from 7942, 6803, and 7002.

- 7002 has higher amounts of unsaturated fatty acids in its membrane.

Construct 7002 mutants:

- *7002ΔdesB*
- *7002ΔdesE*
- *7002ΔdesF*



Comparative Genomics: Desaturases

7942		6803		7002	
Locus	**Description**	**Locus**	**Description**	**Locus**	**Description**
2561	delta-9 acyl-phospholipid desaturase	2538	acyl-CoA desaturase, desC	A2198	delta-9 acyl-lipid desaturase, desC
		1594	fatty acid desaturase, desA	A2756	homology to SYNPCC70025_A0159, desA
		1727	delta 15 desaturase, desB	A0159	omega-3 acyl-lipid desaturase, desB
		1931	delta-6 desaturase, desD	A1989	syn-2, delta 9 acyl-lipid fatty acid desaturase, desF
				A2833	fatty acid desaturase, desE

Mechanisms of Biofuel Tolerance: Efflux Pumps

Comparative Genomics: Efflux Pumps

7942		6803		7002	
Locus	**Description**	**Locus**	**Description**	**Locus**	**Description**
1869	cation efflux system protein	1991	cation or drug efflux system protein	A0587	cation efflux system protein CzcA
1938	multidrug-efflux transporter	1260	quinolene resistance protein NorA	A0589	arsenite efflux pump ACR3
2032	multidrug-efflux transporter quinolene resistance protein NorA	1494	cation or drug efflux system protein, AcrB, TtgB, MexF BLAST hit	A0087	major facilitator transporter
2369	hydrophobe/amphiphile efflux-1 HAE1, AcrB, TtgB, MexF BLAST hit	2483	Probable multidrug resistance protein norM (Multidrug-efflux transporter)	A1013	hydrophobe/amphiphile efflux-1 (HAE1) family protein, AcrB, TtgB, MexF BLAST hit
1989	cation diffusion facilitator family transporter	2125	cation or drug efflux system protein	A1574	RND family efflux transporter MFP subunit
1699	MATE efflux family protein	2737	cation or drug efflux system protein	A2463	cation efflux system protein
792	multidrug efflux MFS transporter	3105	cation or drug efflux system protein	A2552	RND family efflux transporter MFP subunit
				A0585	Outer membrane efflux protein
				A0591	RND family efflux transporter MFP subunit
				A0719	multidrug efflux transporter
				A1483	RND family efflux transporter MFP subunit

Construct 7002 mutants:

- 7002ΔA1013
- 7002ΔA0585
- 7002ΔA0719

Mechanisms of Biofuel Tolerance: ROS-Degrading Proteins

Comparative Genomics: ROS-Degrading Proteins

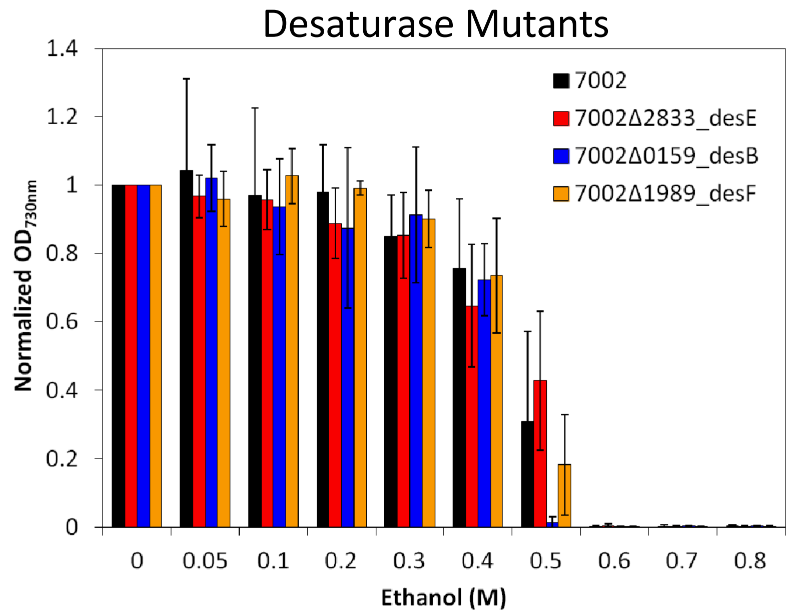
7942		6803		7002	
Locus	Description	Locus	Description	Locus	Description
801	superoxide dismutase	1451	superoxide dismutase, sodB	A0242	Mn-superoxide dismutase, sodB
1214	glutathione peroxidase	1769	glutathione peroxidase	A0117	glutathione peroxidase
1656	catalase/peroxidase HPI	1399	catalase HPI, katG	A2422	catalase/peroxidase HPI, katG
1937	peptide methionine sulfoxide reductase	46	methionine sulfoxide reductase A	A0215	methionine sulfoxide reductase A
2190	methionine sulfoxide reductase B	218	methionine sulfoxide reductase B	A0672	methionine-R-sulfoxide reductase
437	glutathione peroxidase	1305	glutathione peroxidase	A0970	glutathione peroxidase
B2620	putative catalase	239	methionine sulfoxide reductase A (protects against oxidative stress)		

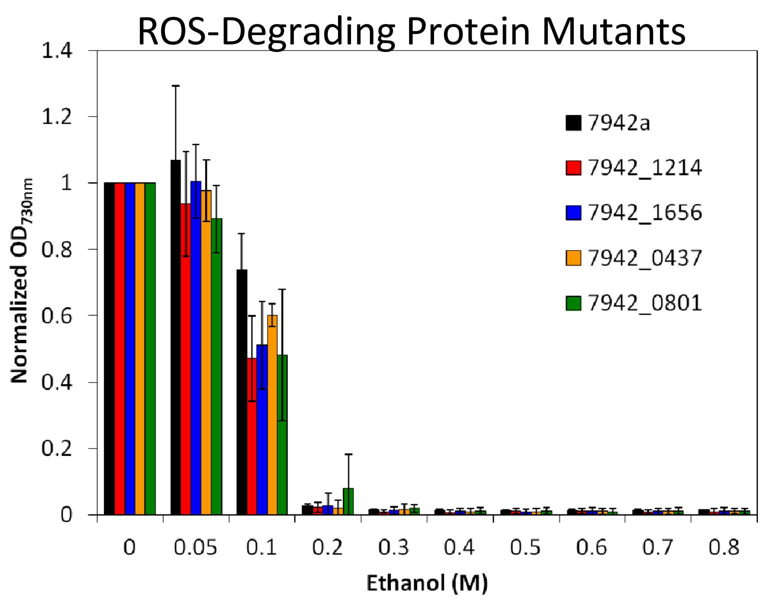
7942 overexpression mutants

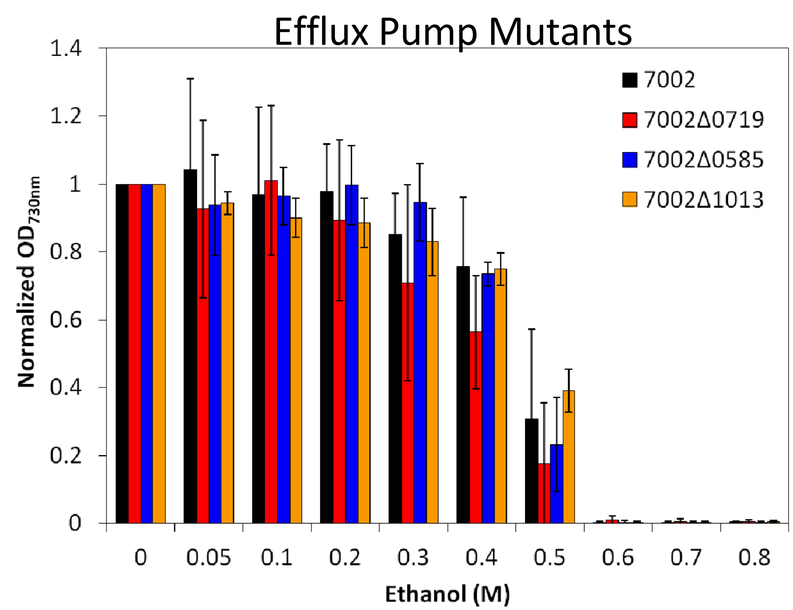
- 7942_1214
- 7942_1656
- 7942_0437
- 7942_0801

Ethanol Growth Inhibition of Mutants

- 7002Δ*desB* had reduced ethanol tolerance
- No significant change in biofuel tolerance for the efflux pump mutant and ROS-degrading protein mutants







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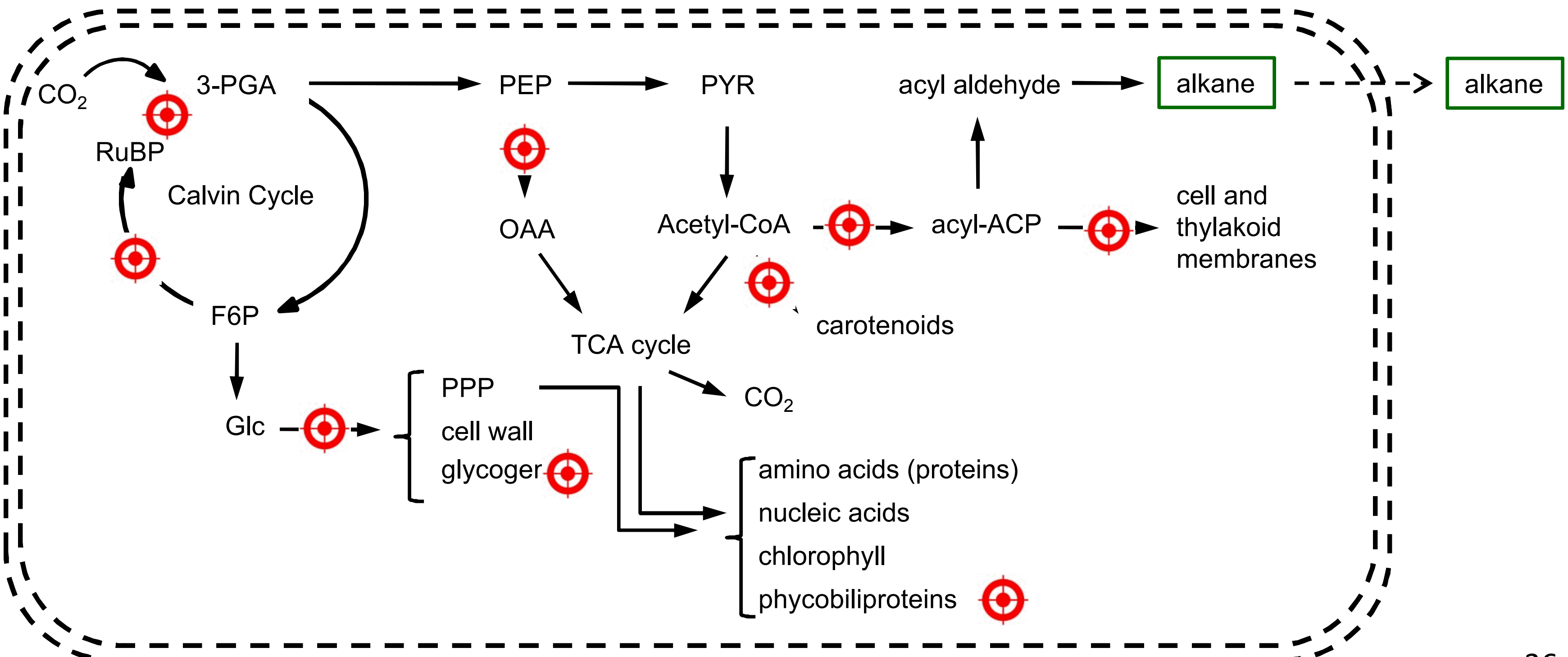
Conclusions

- Advancements in engineering cyanobacteria for FFA production
  - Successful FFA production and excretion in two cyanobacterial hosts
  - Cloning and expression of green algal genes for FFA synthesis
  - Investigation of inducible and native promoters for gene expression
- Characterization of the effects of FFA production in cyanobacteria
  - Physiological effects: cell growth, stress, cell death, photosynthetic yield, photosynthetic pigments
  - Identification of target genes affecting cell physiology during FFA production (RNA-seq, mutants)
- Host strain selection and characterization
  - Minimal physiological effects of FFA production in 7002 at 30°C
  - Degree of membrane saturation is important for biofuel tolerance.

Future Work

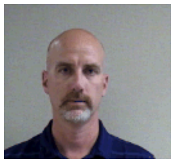
Early Career LDRD: Systems-Level Synthetic Biology for Advanced Biofuel Production

- *Synechococcus* sp. PCC 7002
- Improve cyanobacterial alkane biosynthesis
- Objective: To develop a metabolic engineering method for parallel modification of multiple genetic targets in cyanobacteria for rapid strain development



Acknowledgements

Hyperspectral Imaging



Howland D.T. Jones

Michelle Raymer

Omar F. Garcia

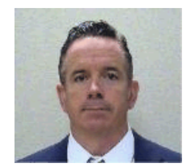
Biofuel Toxicity Experiments

Christine Trahan

Project Mentors / Managers

Anthony Martino

Eric Ackerman



Jim Carney

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Kansas State University

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