SAND2011-6635P

Engineering Green Fuel:

Genetic Engineering of Cyanobacteria for the Production of Biodiesel Feedstocks

Anne M. Ruffing

Truman Fellow

Sandia National Laboratories

September 21, 2011

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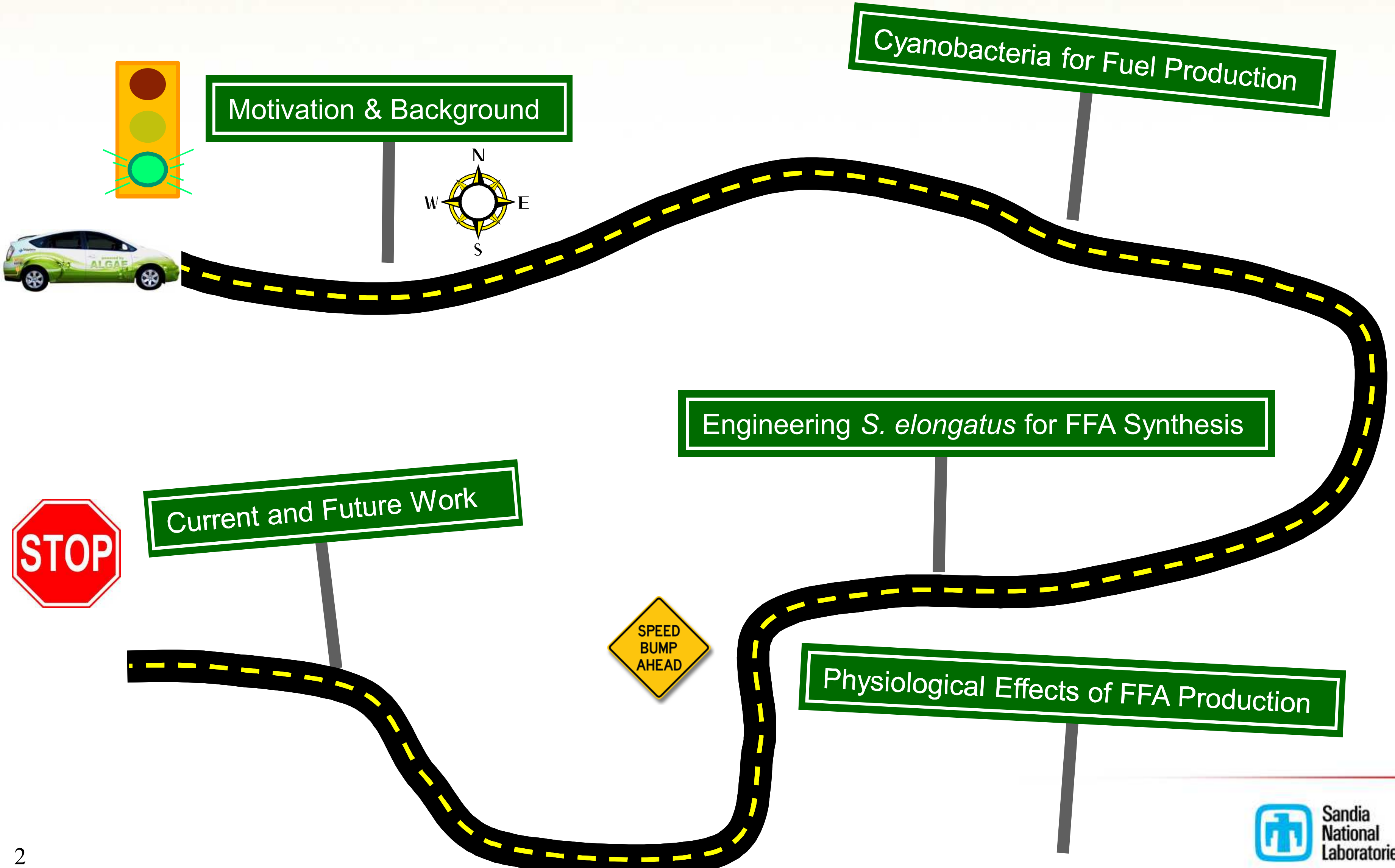
Roadmap to Cyanobacteria-Based Fuels



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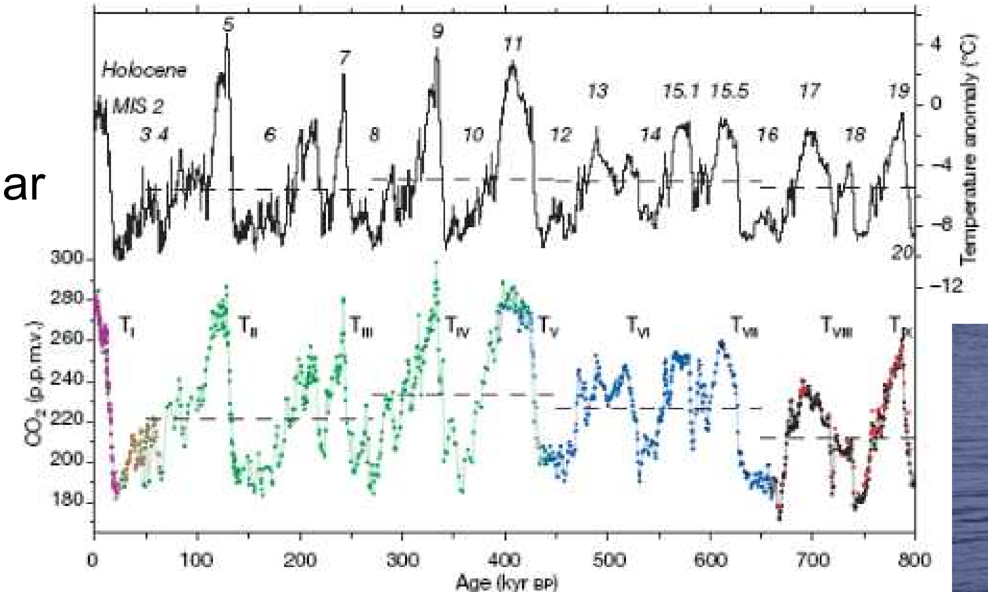
Humanity's Top 10 Problems for the Next 50 Years

- Richard E. Smalley 2003

1. **Energy**
2. Water
3. Food
4. Environment
5. Poverty
6. Terrorism & War
7. Disease
8. Education
9. Democracy
10. Population

BP gulf oil spill - 2010



Luthi D. Nature. 453, 379-382.

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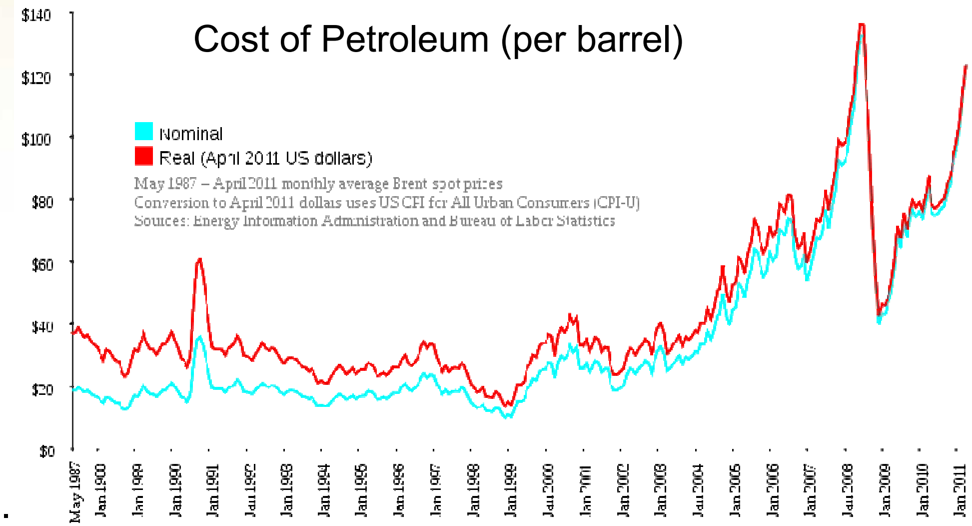

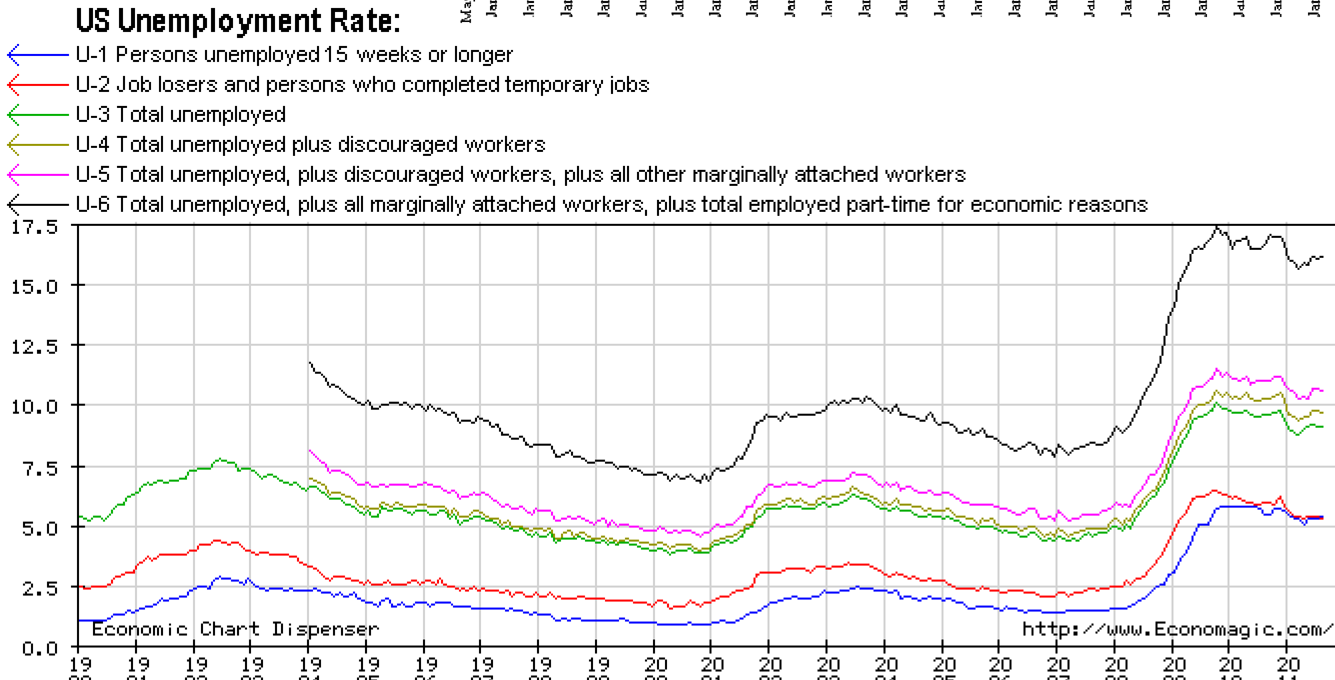

http://www.economagic.com/

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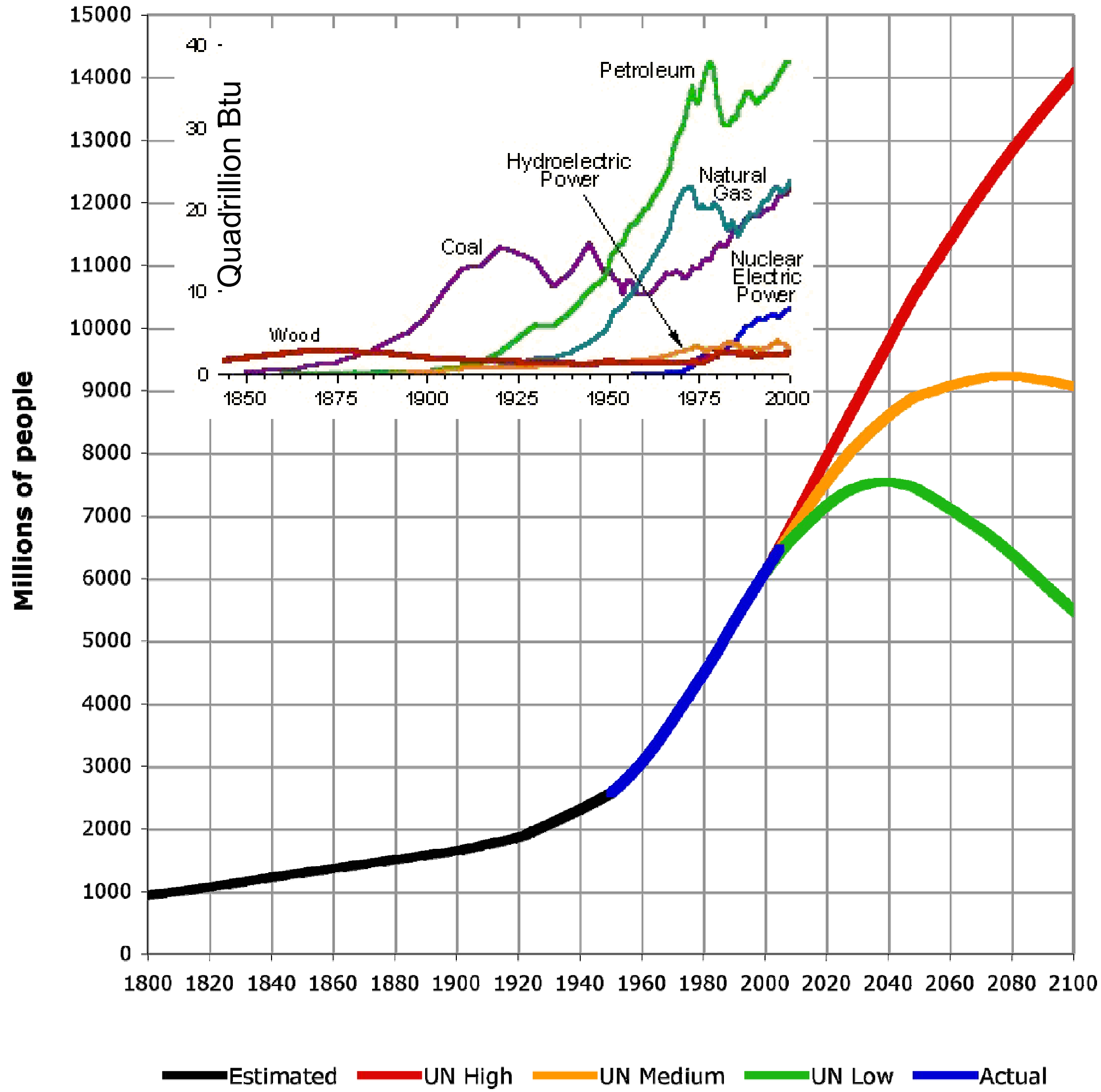
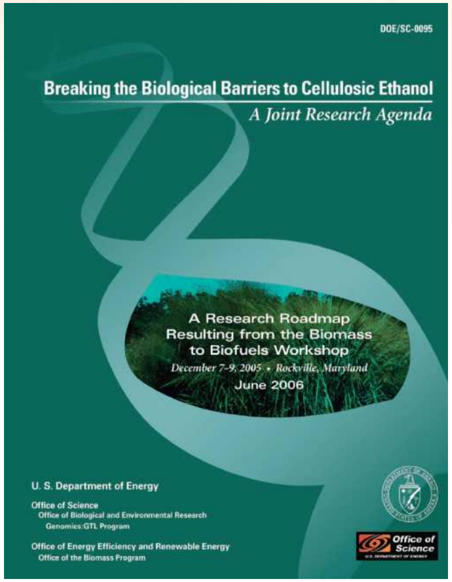
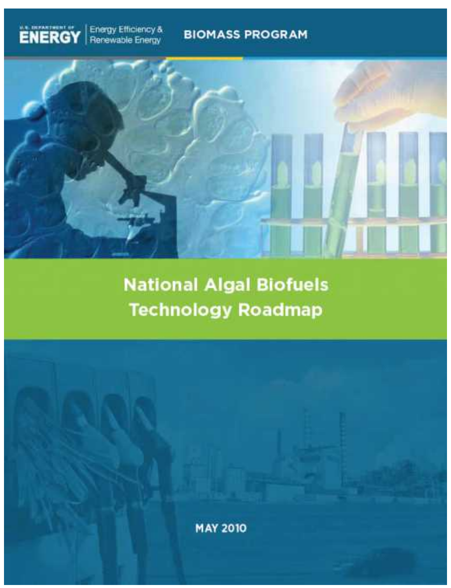


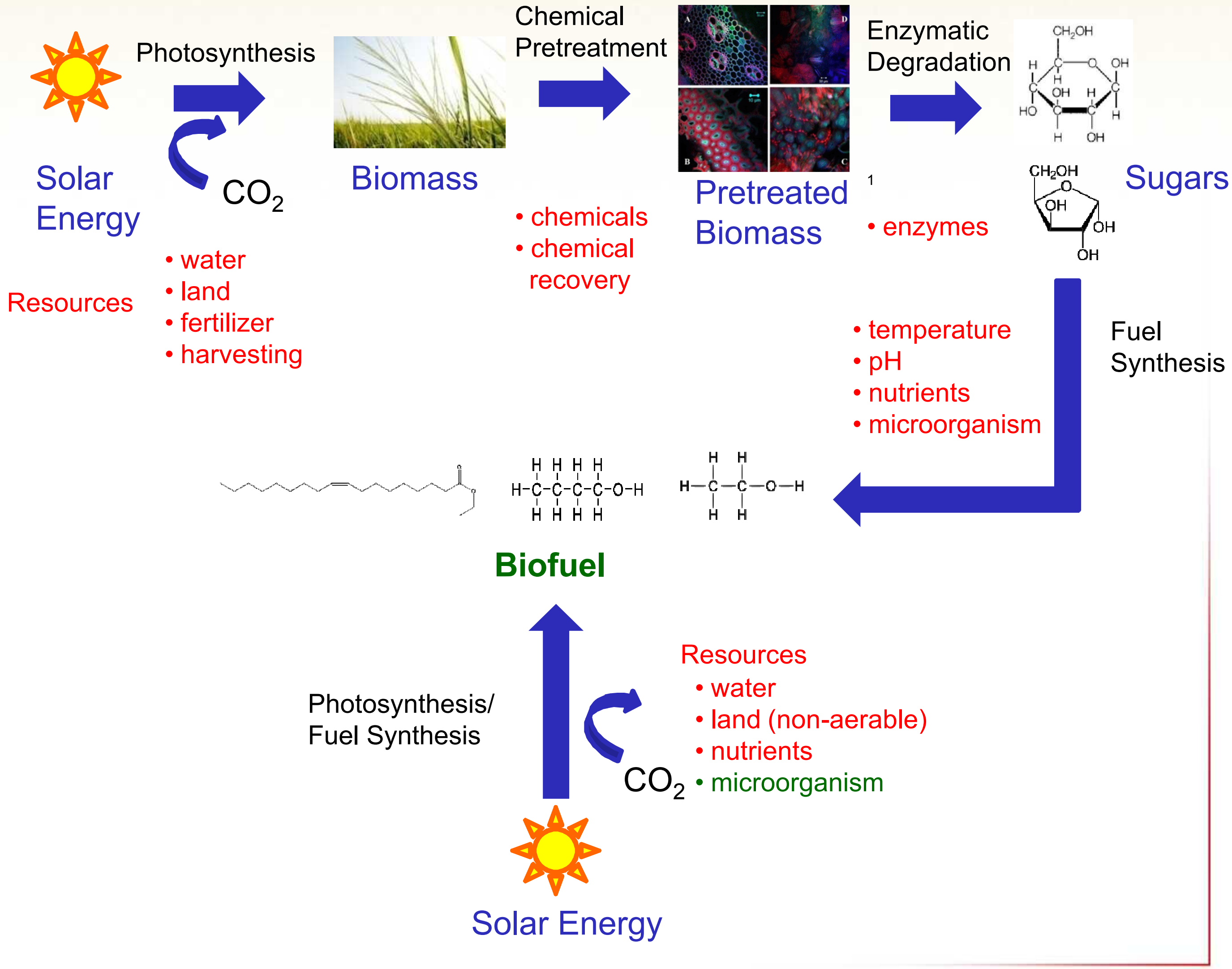
Biofuels: A Solution to the Energy Problem?

Biomass Feedstocks



Photosynthetic Microorganisms





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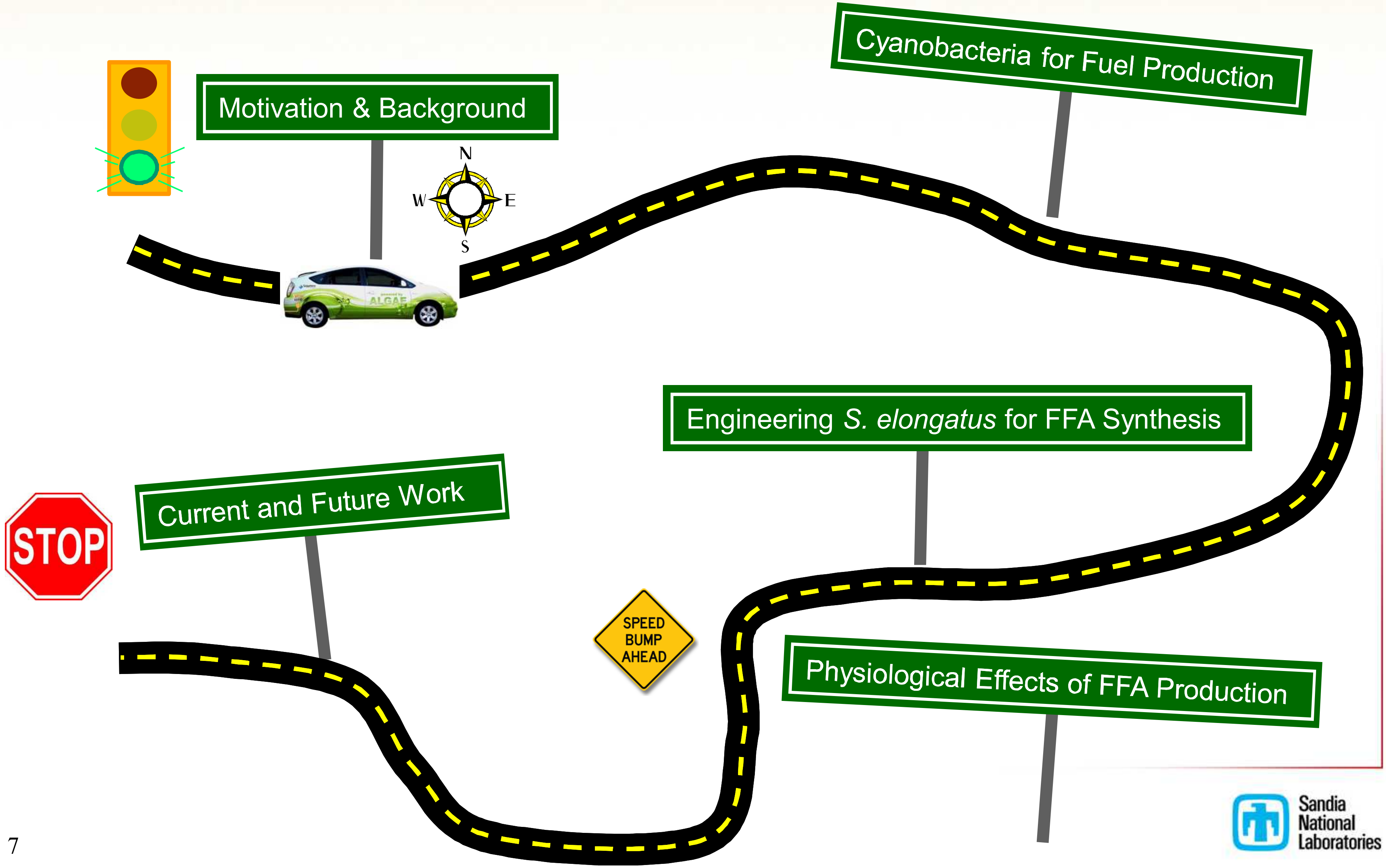
1. Singh S, Simmons B, Vogel K. (2009) J. of Biotech. & Bioeng. 104 (1): 68-75.

Roadmap to Cyanobacteria-Based Fuels



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Cyanobacteria: An Evolutionary Perspective

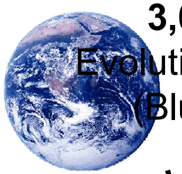
Earth's History

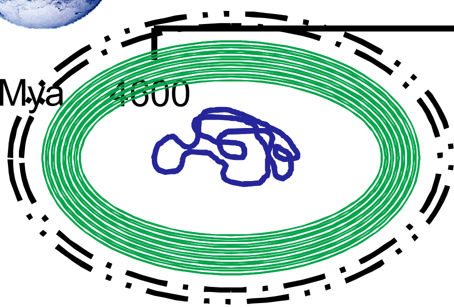
3,000,000,000 ya
Evolution of Cyanobacteria
(Blue-Green Algae)

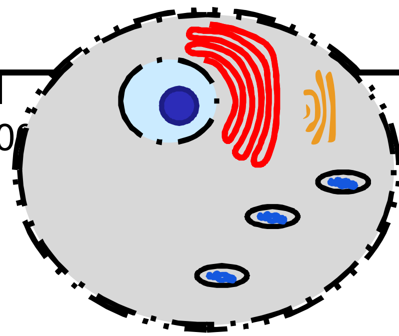
1,500,000,000 ya
Evolution of Eukaryotic Algae

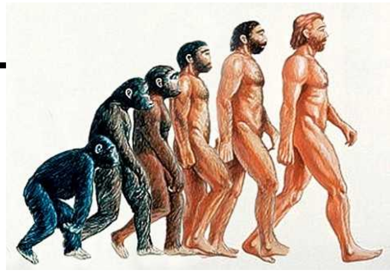
500,000 - 200,000 ya
Evolution of *Homo sapiens*

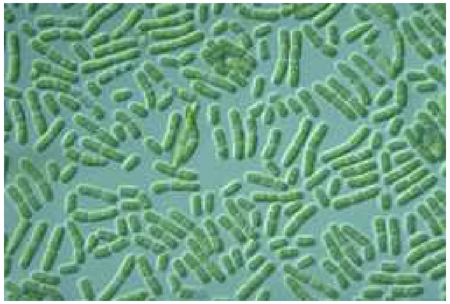
Mya 4600 | 3000 | 1500





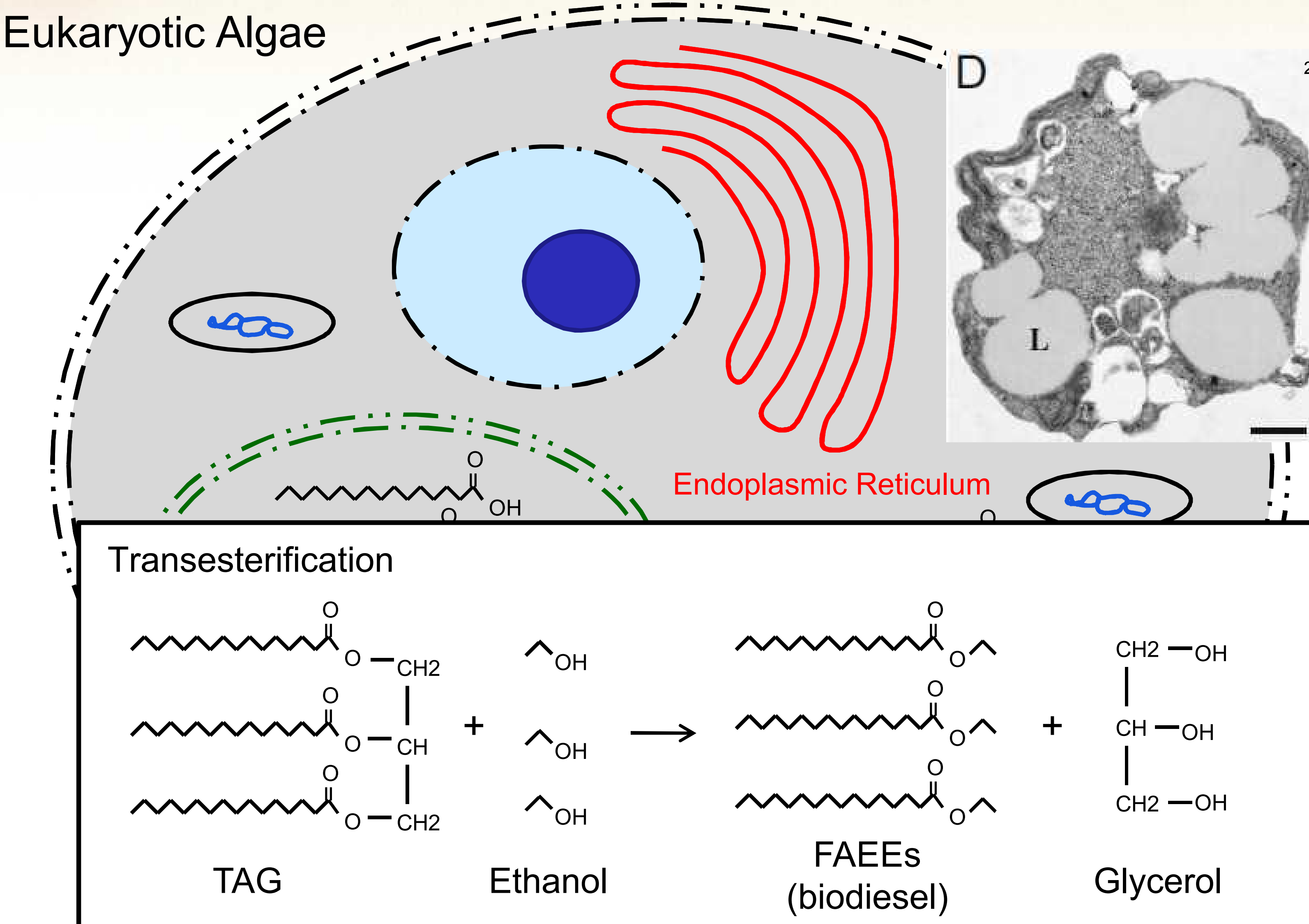




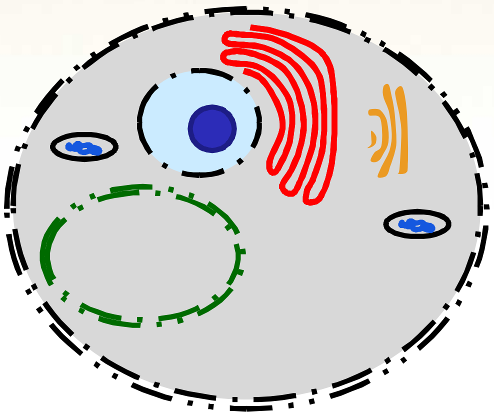


1. Photosynthesis: source of cellular energy (sunlight)
2. Carbon Fixation: carbon source = CO_2 (greenhouse gas)

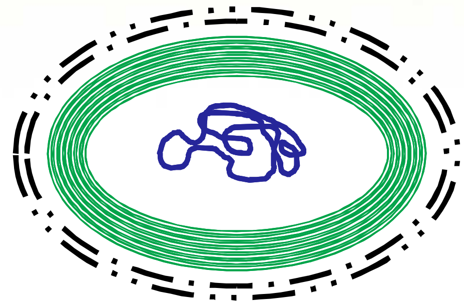
Why Cyanobacteria?



Why Cyanobacteria?



Eukaryotic Algae



Cyanobacteria

Genetic manipulation is difficult	Genetic manipulation is straightforward
Multiple membrane barriers	Naturally transformable (model strains)
Few genetic tools available	Genetic tools developed since 1980's
Gene expression and gene knockouts have proven to be difficult	Many successful examples of genetic manipulation and multiple mutations

Genetic Engineering: Tailor-design biocatalysts for fuel production

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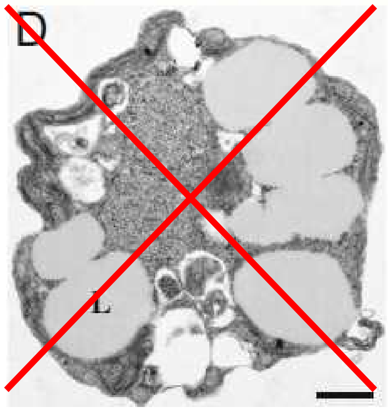
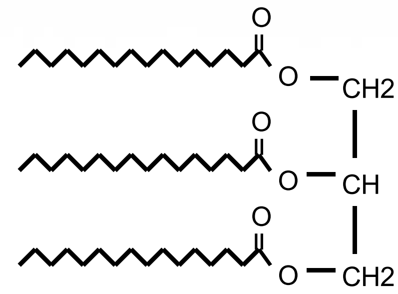
Downstream Processing: Fuel Recovery

Eukaryotic Algae



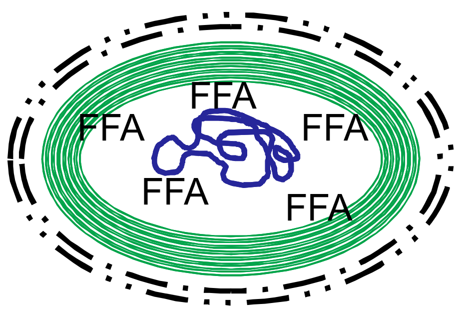
Dewatering
(centrifugation, flocculation)

TAG Extraction
(solvent)



TAG

Cyanobacteria



Why Not Cyanobacteria?

Possible Advantages of Cyanobacteria-Based Fuel

1. Fast growth rates compared to plants (similar to algae)
2. Harvest energy from sunlight (renewable)
3. Reduce greenhouse gas emissions (carbon fixation)
4. Fuel secretion - simplified downstream processing and continuous cultivation (reduced operational costs)
5. Potential for greater fuel production and other desirable traits for large scale processing (genetic engineering)
    - Prevent ‘escape’ of GMO’s
    - Reduce contamination
    - Improve rate of photosynthesis
    - Increase light penetration
    - Enhanced CO_2 uptake
    - Temperature resistance

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Engineering Cyanobacteria for Biofuel Production

Fuel Product	Amount (g/L)	Cyanobacterium	Reference
Ethanol	0.23	Synechococcus elongatus PCC 7942	Deng, 1999
	0.55	Synechocystis sp. PCC 6803	Dexter, 2009
	~ 2.5	Synechocystis sp. PCC 6803; Anabaena sp. PCC 7120	Algenol (2010 Patent)
Ethylene	0.016	Synechococcus elongatus PCC 7942	Sakai, 1997
	~ 5	Synechococcus elongatus PCC 7942	Takahama, 2003
Isobutyraldehyde / Isobutanol	1.1 / 0.45	Synechococcus elongatus PCC 7942	Atsumi, 2009
Isoprene	~ 0.0005	Synechocystis sp. PCC 6803	Lindberg, 2010
Free fatty acids	0.007	Synechocystis sp. PCC 6803; Synechococcus elongatus PCC 7942	Kaczmarzyk, 2010
	0.141	Synechocystis sp. PCC 6803; Synechococcus elongatus PCC 7942	Synthetic Genomics (2009 Patent)
N-alkanes	~0.05	Synechococcus sp. PCC 7002; Thermosynechococcus elongatus BP-1	Joule Unlimited (2010 Patent)

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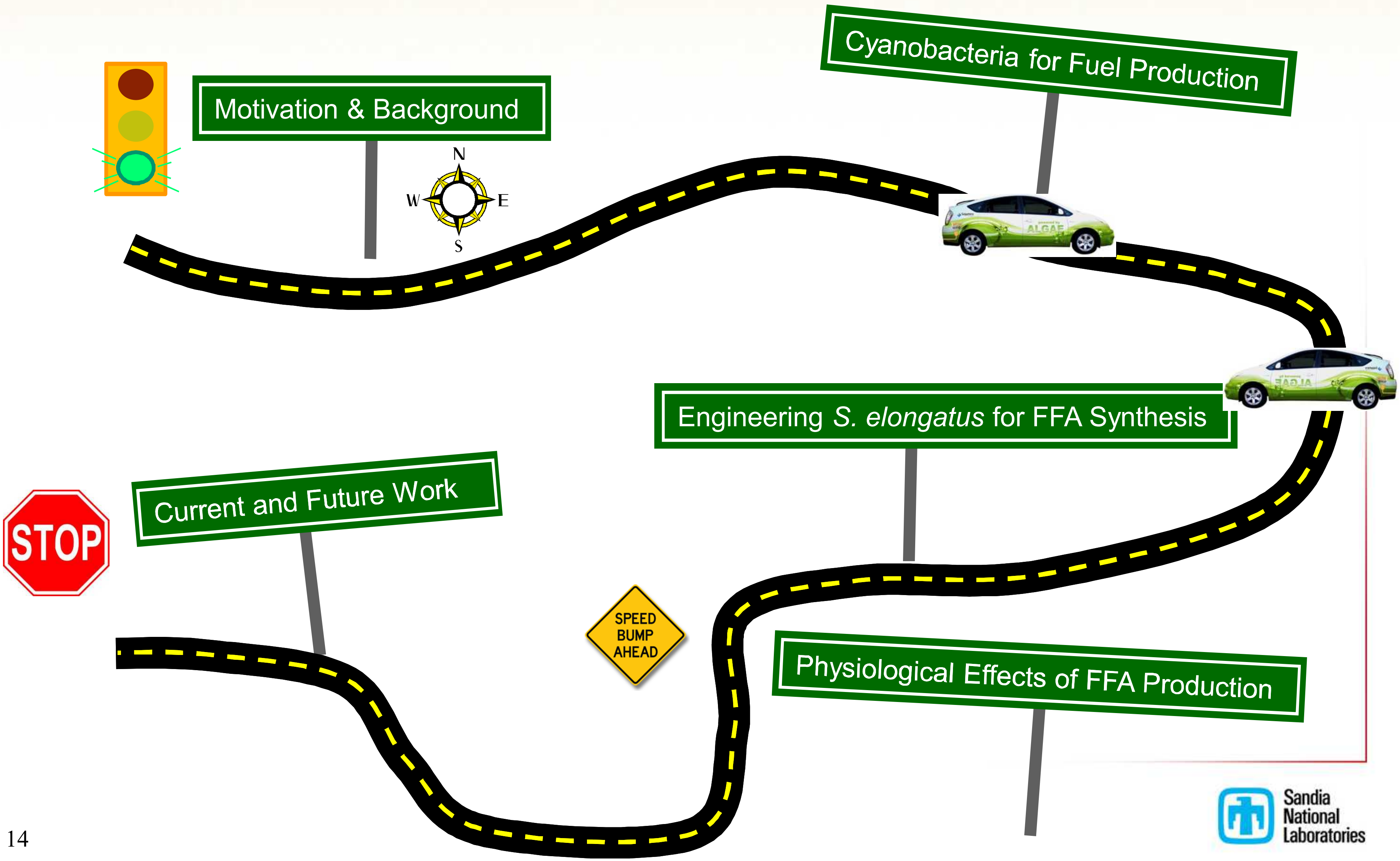
Roadmap to Cyanobacteria-Based Fuels



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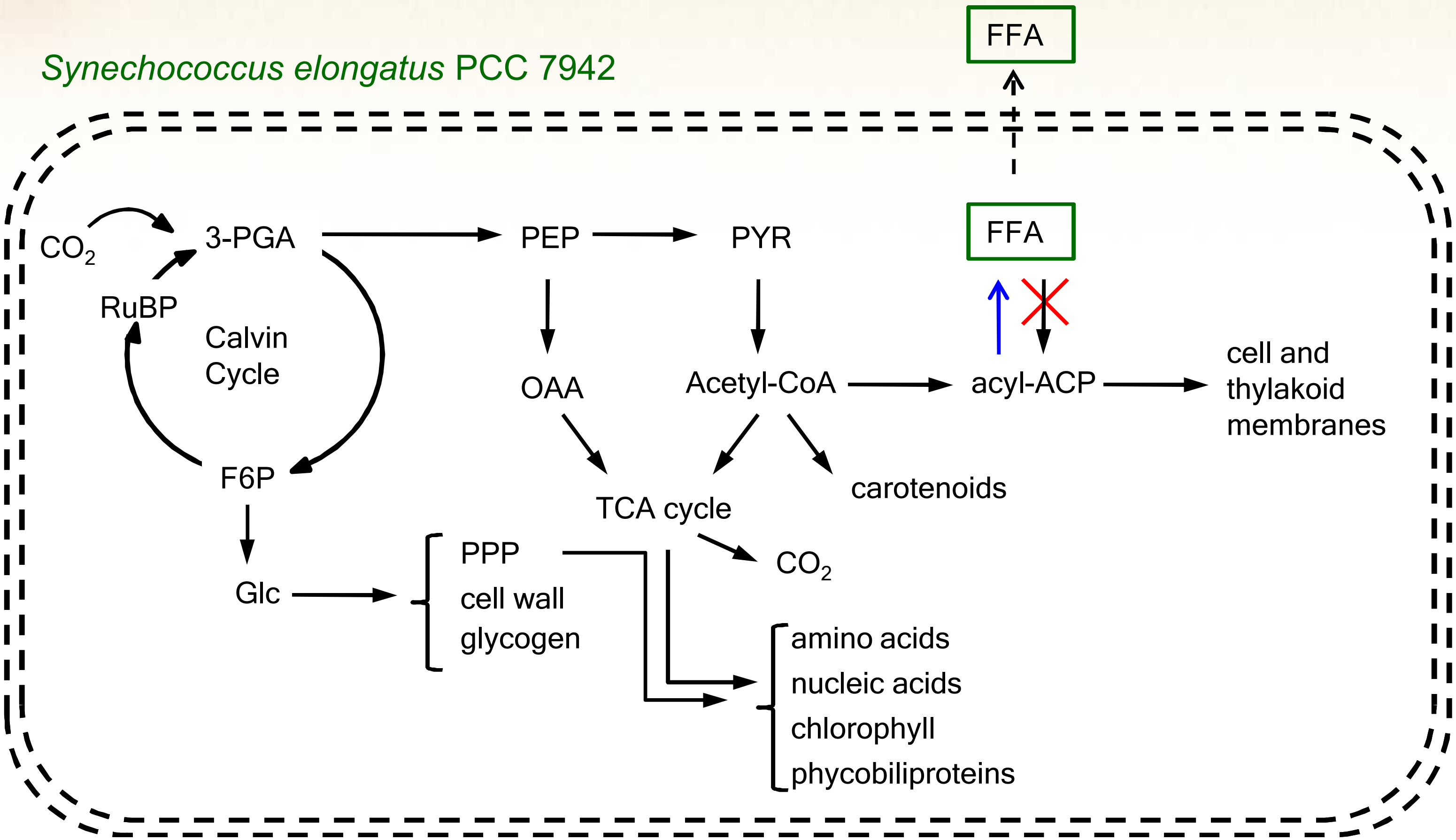
Genetic Engineering of Cyanobacteria to Produce FFA

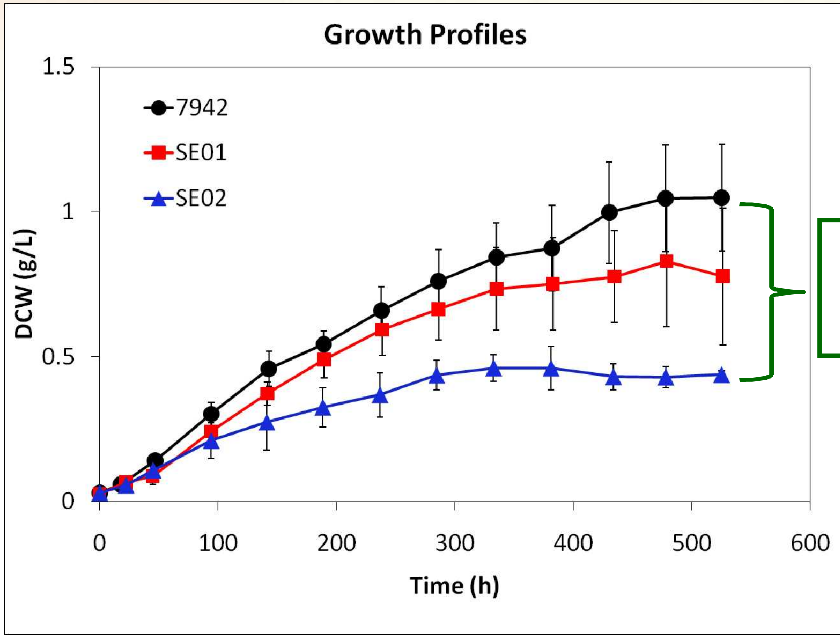


7942: Wild type

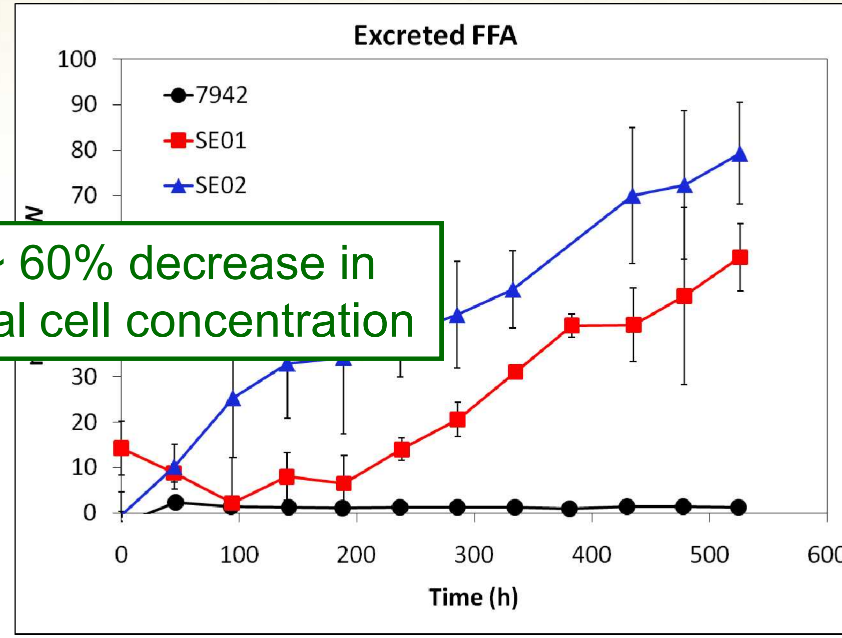
SE01: Knockout of acyl-ACP synthetase

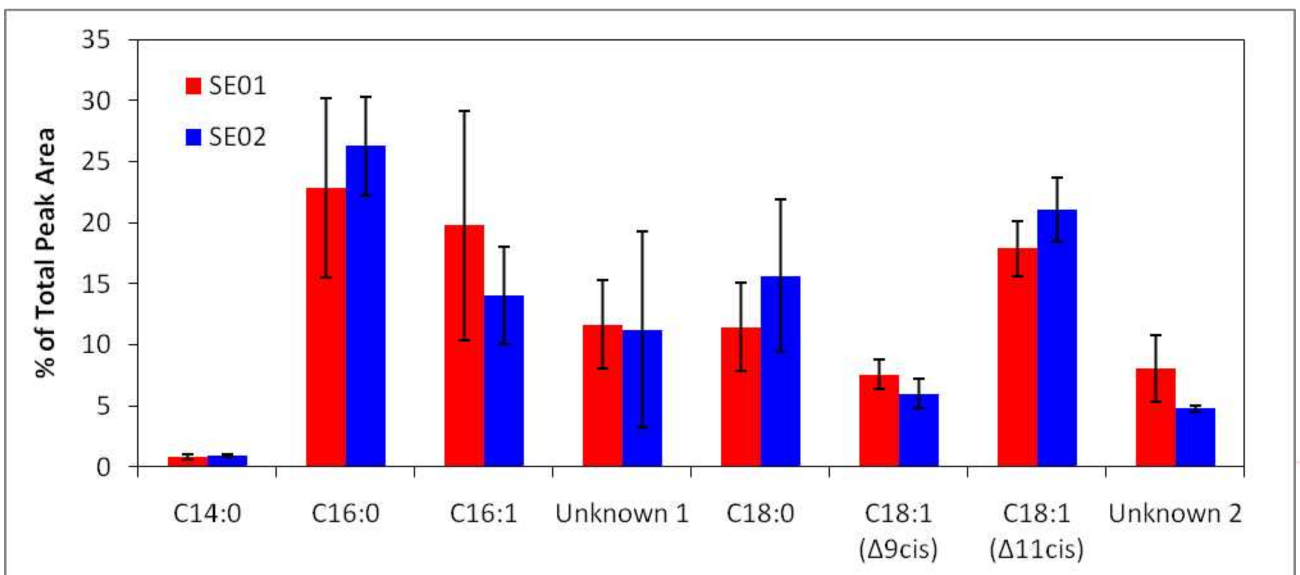
SE02: Knockout of acyl-ACP synthetase; expression of thioesterase ('*tesA* from *E. coli*)

Growth and FFA Production

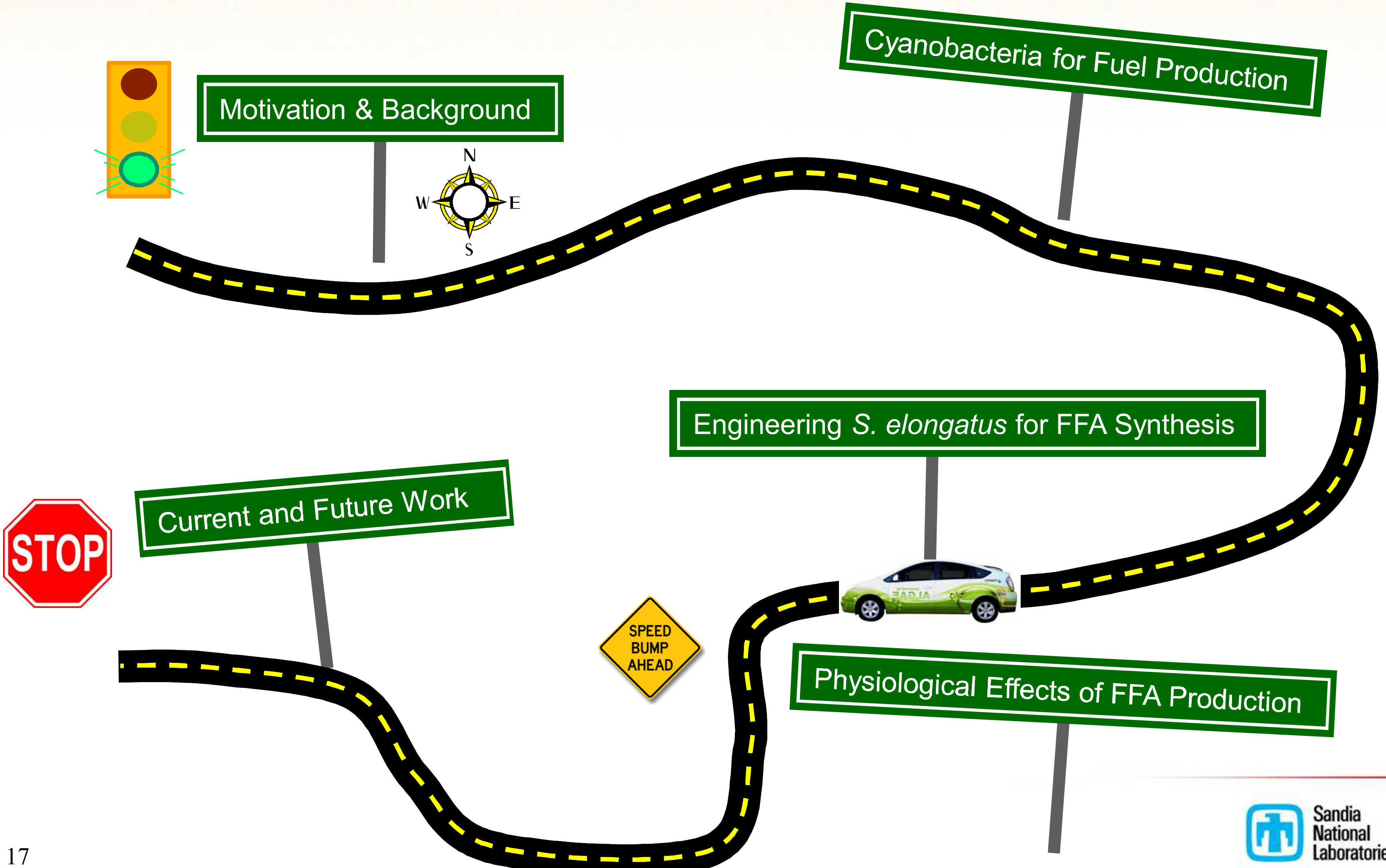


~ 60% decrease in final cell concentration



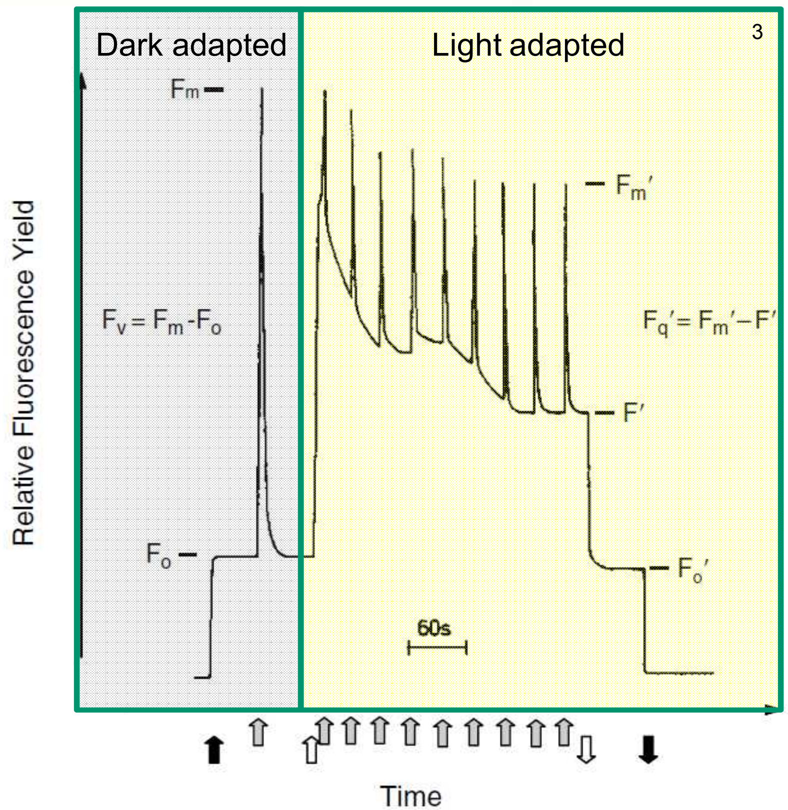


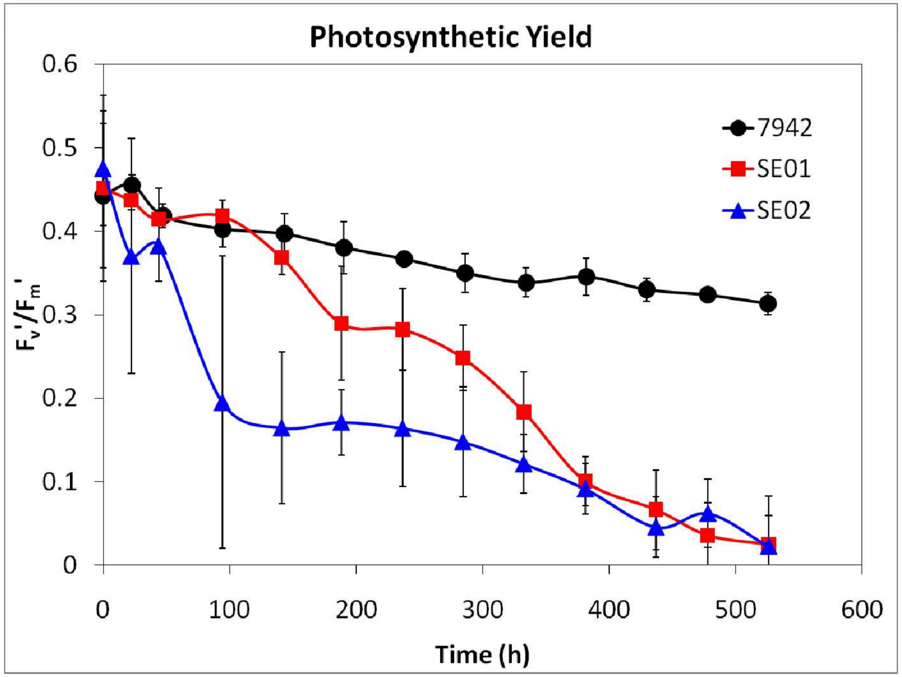
Roadmap to Cyanobacteria-Based Fuels



FFA Production: Effect on Photosynthesis

Pulse Amplitude Modulation (PAM) Fluorescence



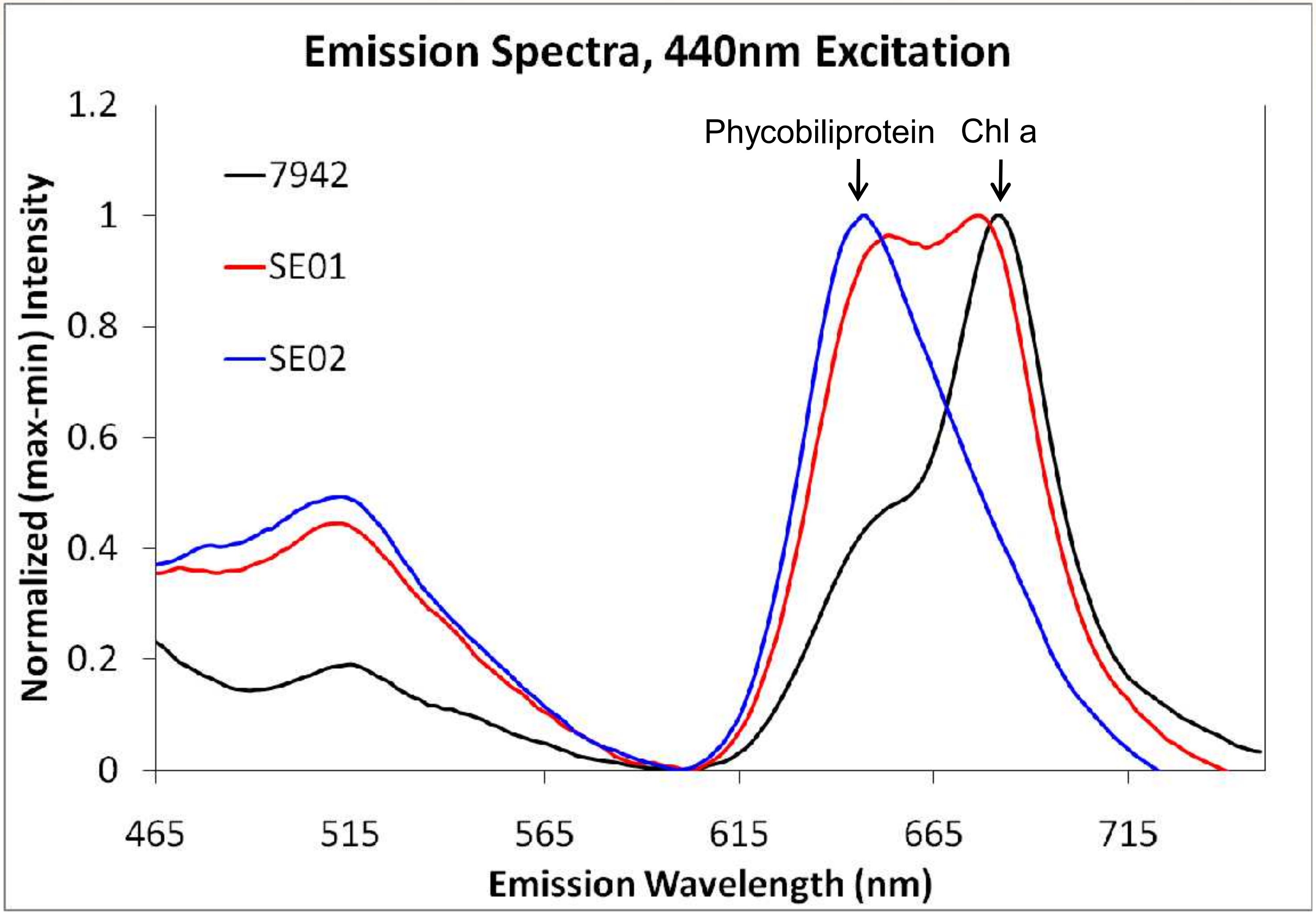


$$\frac{F_v^{'}}{F_m^{'}} = \frac{F_m^{'} - F_0^{'}}{F_m^{'}}$$

Maximum PSII photochemical efficiency or quantum yield in actinic light

3. Cosgrove, J. and M.A. Borowitzka (2010) Chlorophyll a Fluorescence in Aquatic Science. Chapter 1. p1-17.

FFA Production: Effect on Photosynthetic Pigments



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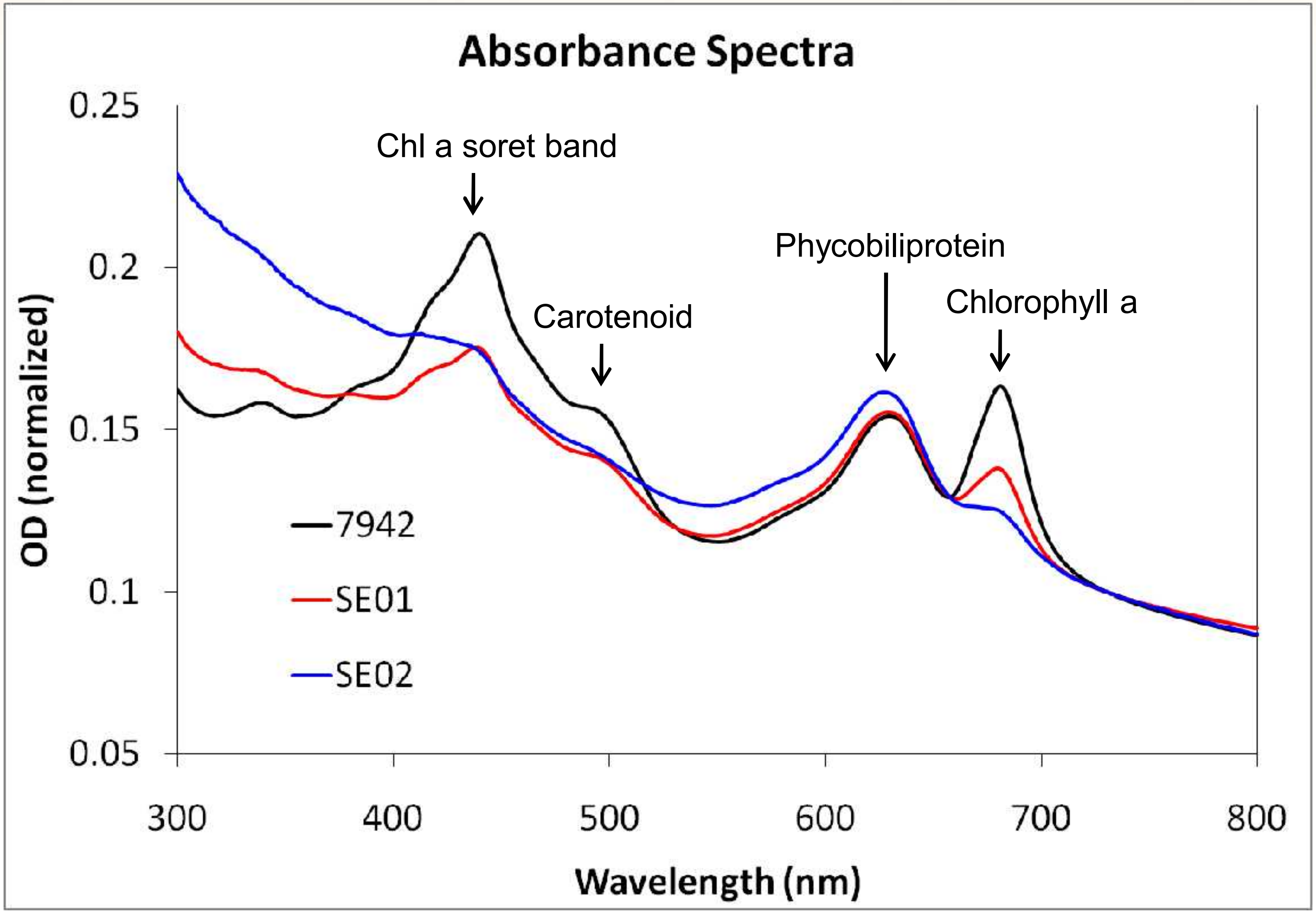
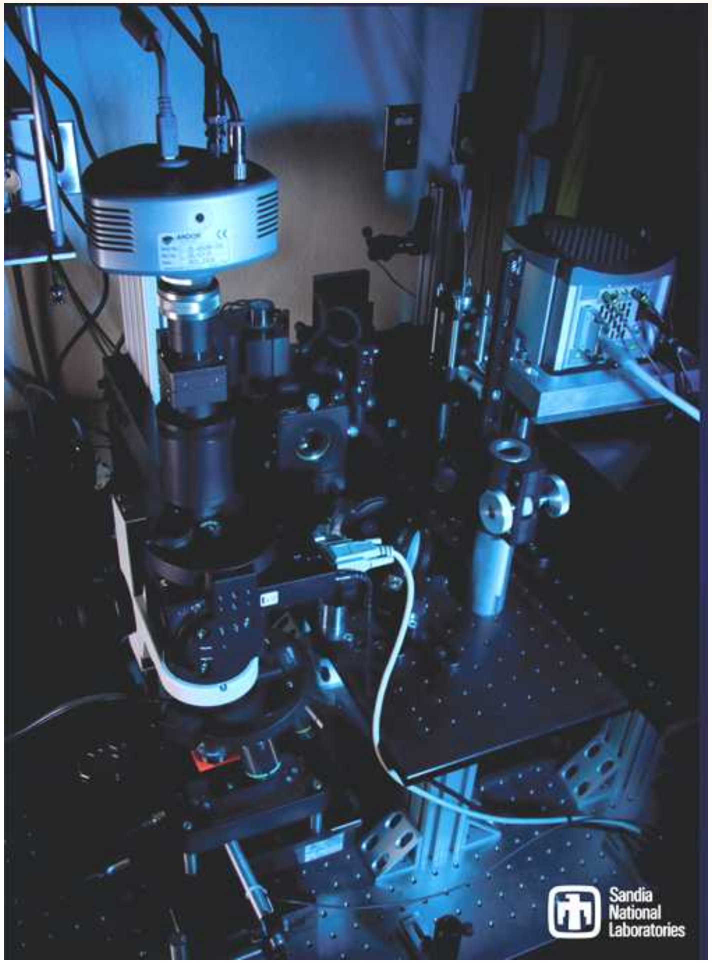
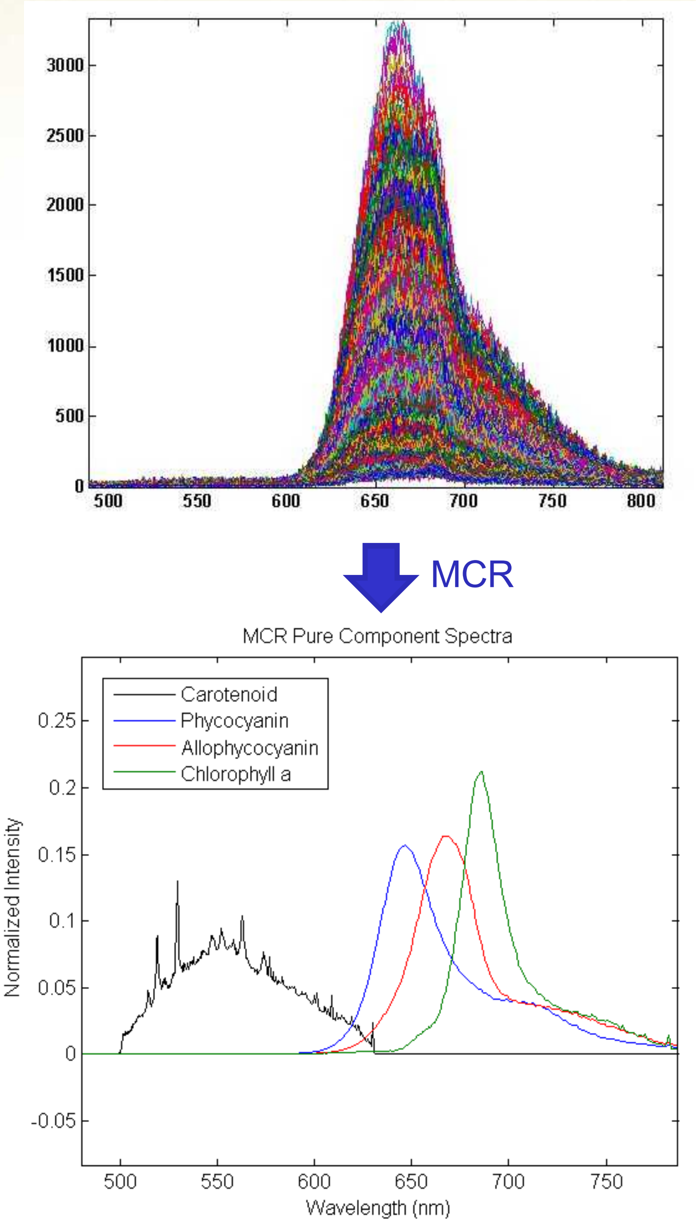


Hyperspectral Confocal Fluorescence Imaging



M. B. Sinclair, D. M. Haaland, J. A. Timlin, and H. D. T. Jones, *Hyperspectral confocal microscope*. (2006). Applied Optics. 45: 6283-6291.



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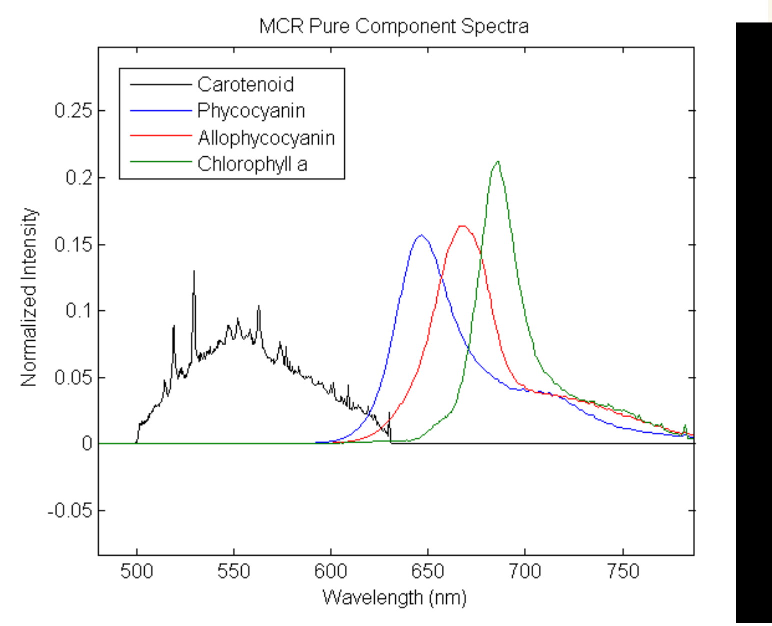
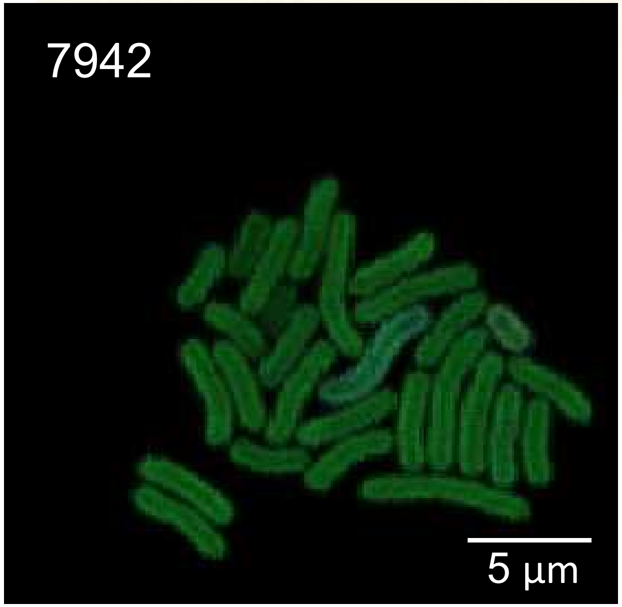
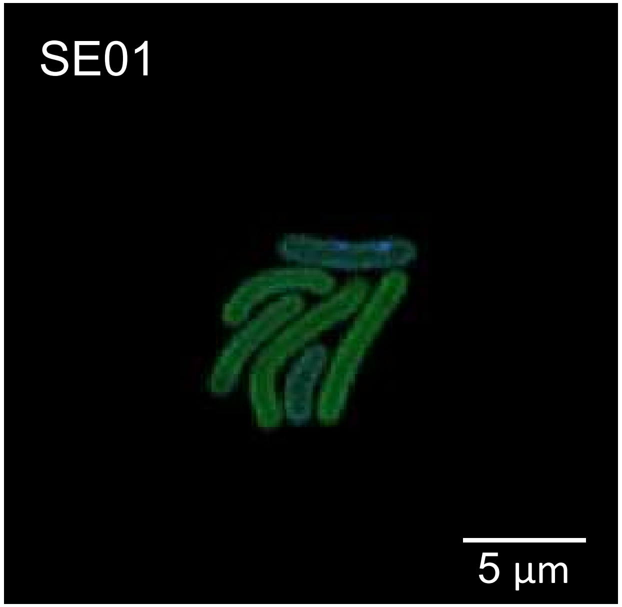
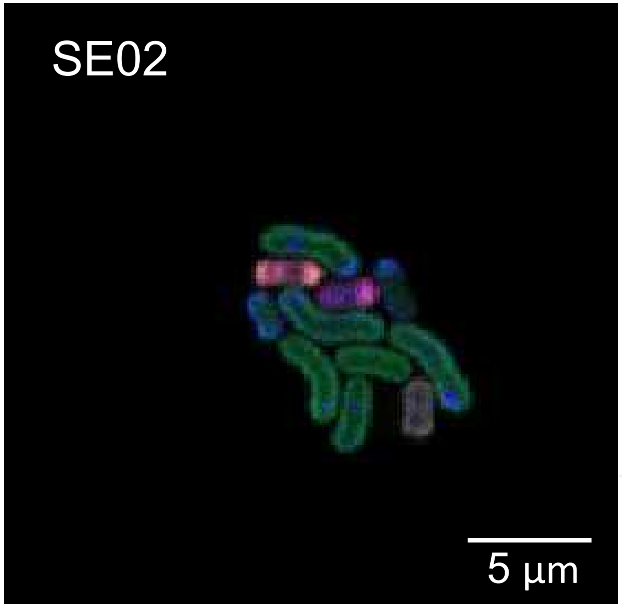
FFA Production: Effect on Thylakoid Membranes









FFA Toxicity

Table 1 Selected bioactivites of various saturated and unsaturated FFAs

Activity	Fatty acid(s)	Key reference
Antimicrobial		
Anti-algal	C8:0, C10:0, C12:0	McGrattan et al. (1976)
	C18:4n-3	Kakisawa et al. (1988)
	C18:1, C18:2, C18:4, C18:5, C20:4, C20:5, C22:6	Arzul et al. (1995)
	C18:2n-6, C18:3n-3	Ikawa et al. (1997)
	C16:0, C18:0, C18:1n-9, C18:2, C18:3n-3, C20:5n-3, C22:6n-3	Wu et al. (2006)
	C16:0, C16:1n-7, C16:1n-7*t*, C16:4n-3, C18:0, C18:1n-9, C18:2n-6, C18:3n-3, C18:4n-3, C20:0, C20:1n-9, C20:4n-6, C20:5n-3, C22:0, C22:1n-9, C22:6n-3	Alamsjah et al. (2008)
Antibacterial (Gram-negative)	C20:4n-6	Knapp and Melly (1986)
	C10:0, C12:0	Bergsson et al. (1998)
	C10:0, C12:0, C14:0, C16:1	Bergsson et al. (1999)
	C15:0, C16:0, C17:0, C18:0, C18:1, C18:4, C20:4, C20:5, C22:0, C22:4, C22:5	Benkendorff et al. (2005)
Antibacterial (Gram-positive)	C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C18:1, C18:2, C18:3	Galbraith et al. (1971)
	C10:0, C12:0, C14:0, C14:1, C16:0, C16:1, C18:1, C18:2, C18:3	Kabara et al. (1972)
	C8:0, C9:0, C10:0, C11:0, C12:0, C13:0, C14:0, C14:1n-5, C16:1n-7, C16:1n-7*t*, C18:2n-6, C18:3n-3, C18:3n-6, C20:1n-9, C20:3n-6, C20:3n-3, C20:4n-6, C22:2n-6, C22:3n-3, C20:4n-6, C22:6n-3	Feldlaufer et al. (1993)
	C16:1n-10	Wille and Kydonieus (2003)
	C15:0, C18:1, C18:4, C20:4, C20:5, C22:0, C22:4, C22:5	Benkendorff et al. (2005)

Desbois AP and Smith VJ. (2010*) Appl. Microbiol. Biotechnol.*. 85: 1629-1642.

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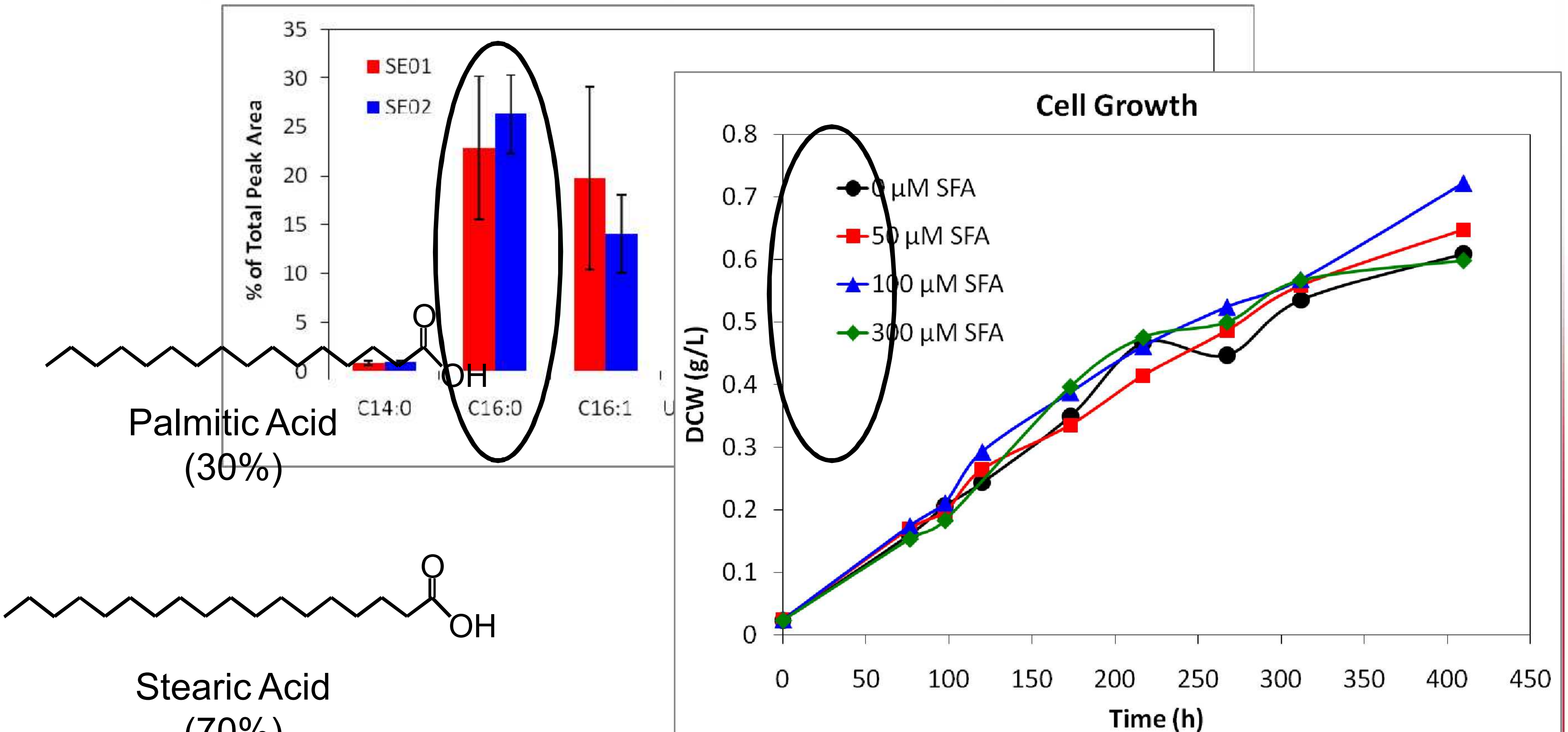
FFA Toxicity: Effect of Saturated FFA



Palmitic Acid
(30%)

Stearic Acid
(70%)

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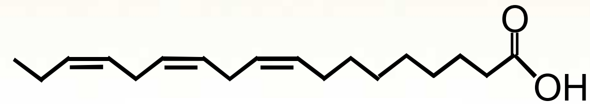
FFA Toxicity: Effect of Unsaturated FFA

0µM 50µM 100µM 300µM

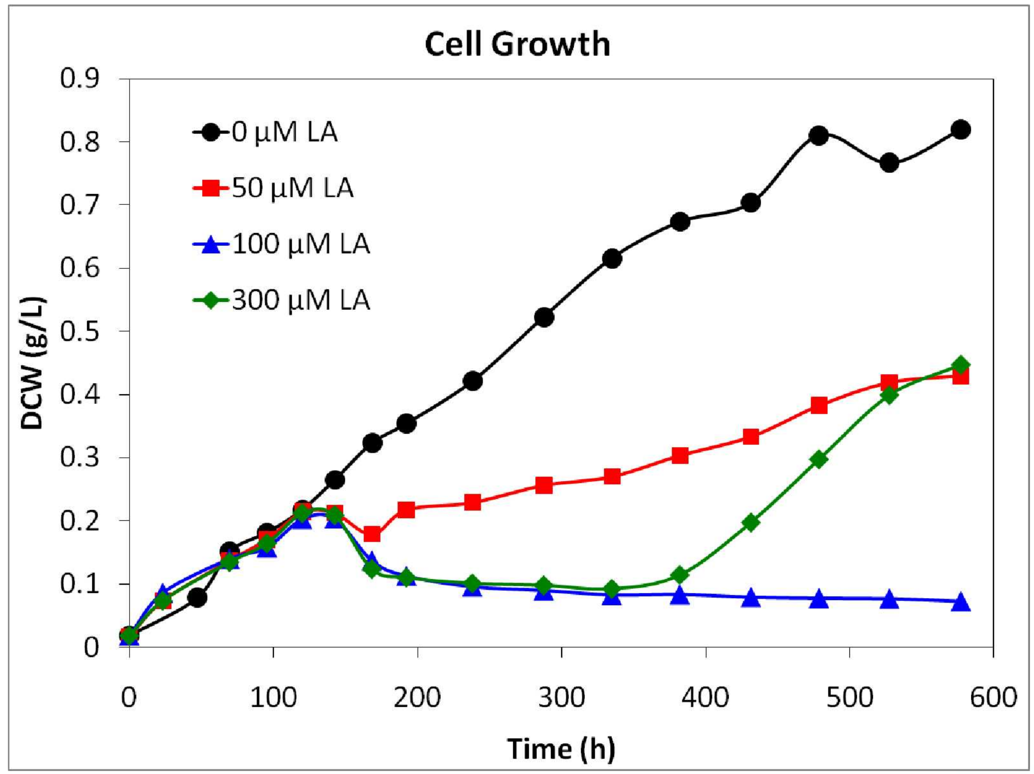
Day 2

Day 3

Day 4



Linolenic Acid



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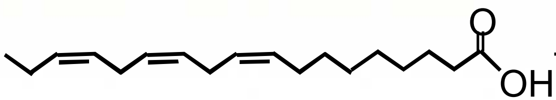
Oxidation of Unsaturated FFA

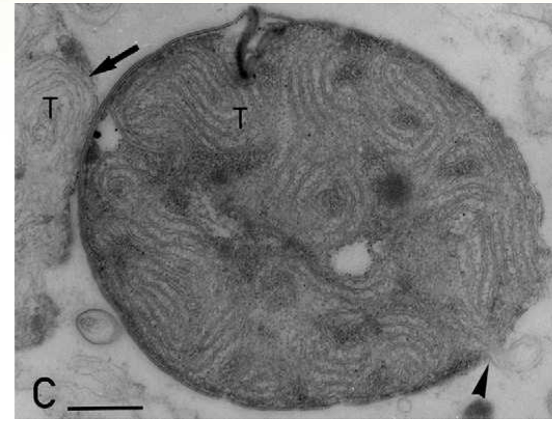


+ O_2 $\xrightarrow{\text{Heat, Light, Metals}}$

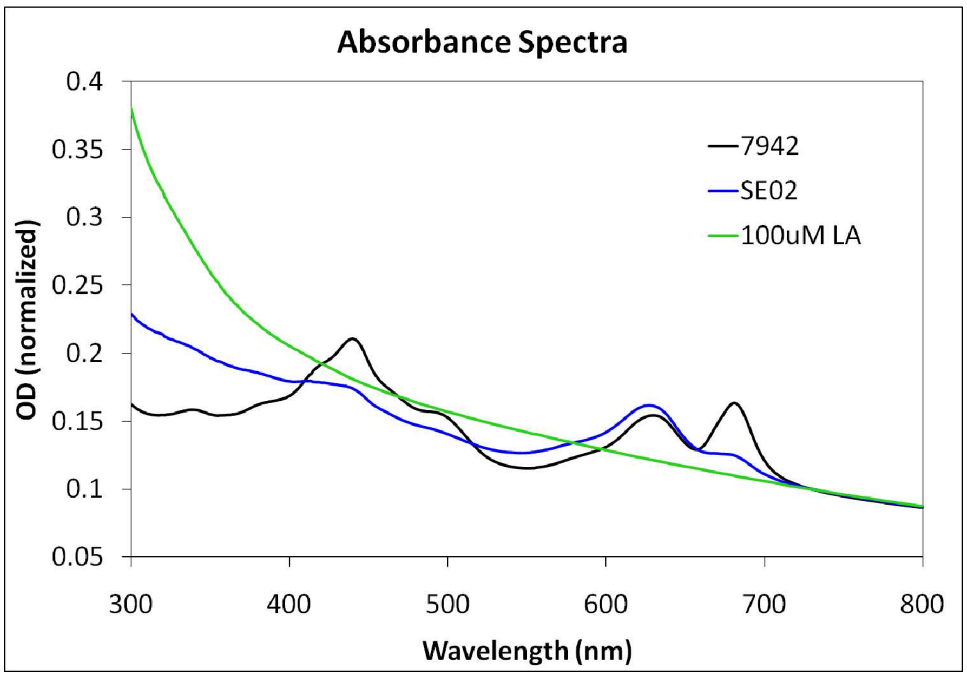
Unsaturated FFA
(linolenic acid)

- Hydroperoxides
- Aldehydes
- Acids
- High MW compounds
- Keto compounds
- Epoxy compounds
- Di and trihydroxy compounds
- Hydroperoxy epidioxides

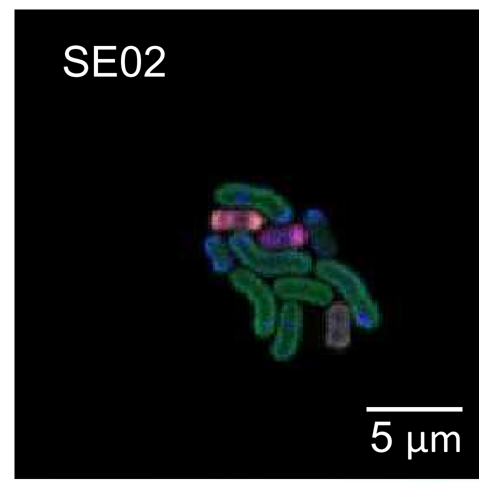
Cyanobacterium: *Anabaena* P9 [2]



0.178 µmol/L α-linolenic acid

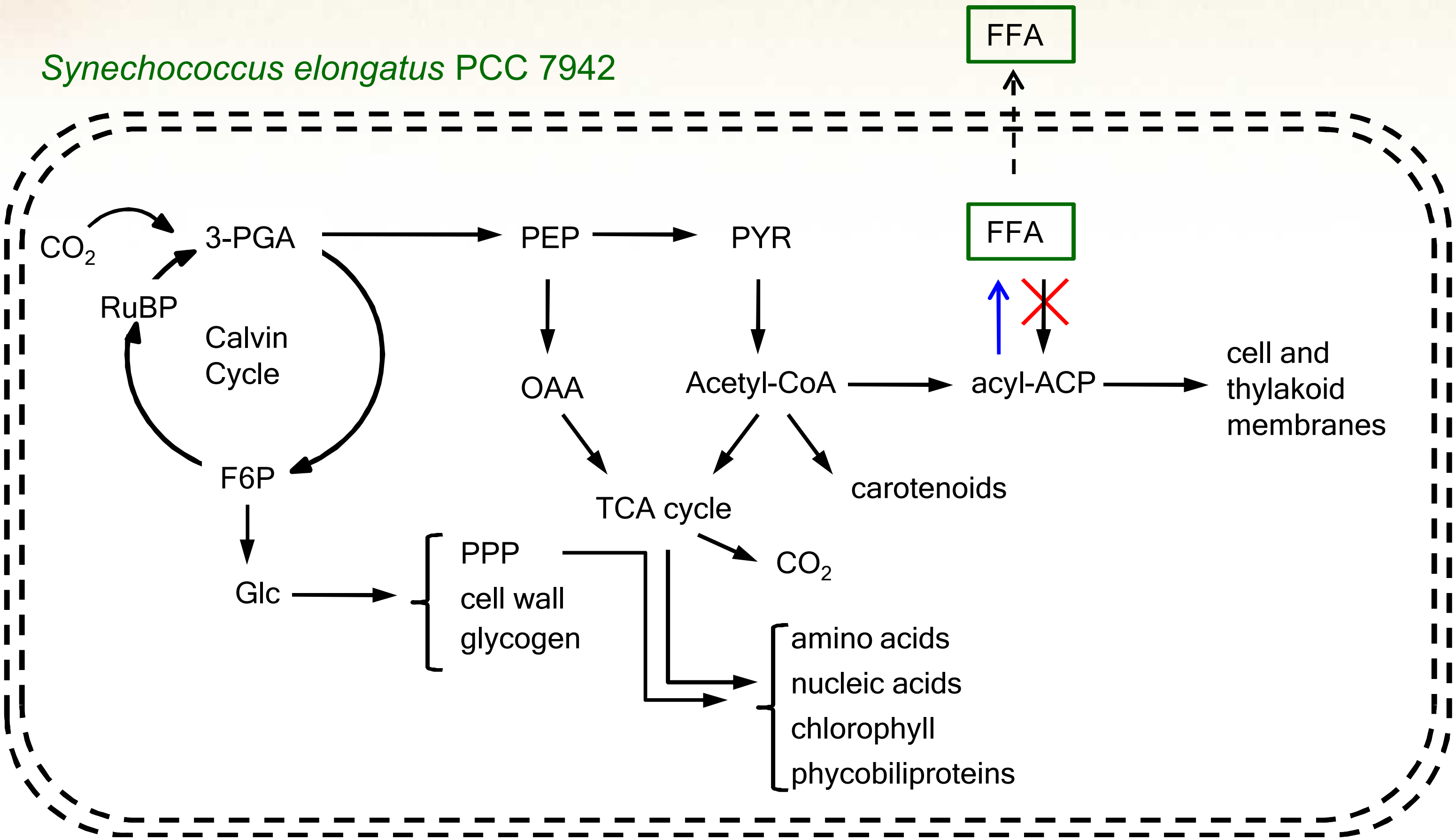


Engineered *S. elongatus* PCC 7942



2. Wu J-T, Chiang Y-R, Huang W-Y, and Jane W-N. (2006) *Aquatic Toxicology*. 80: 338-345.

Physiological Effects Caused by Genetic Engineering?

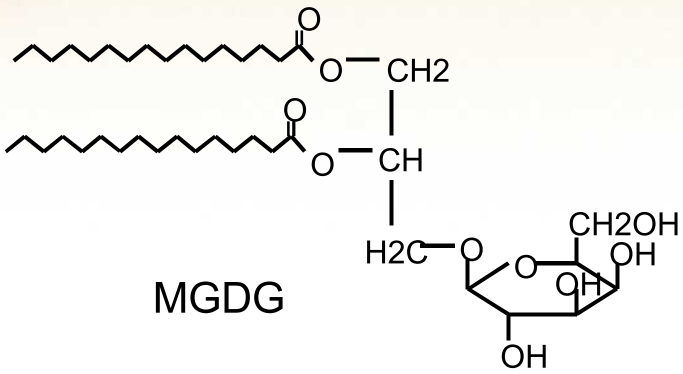


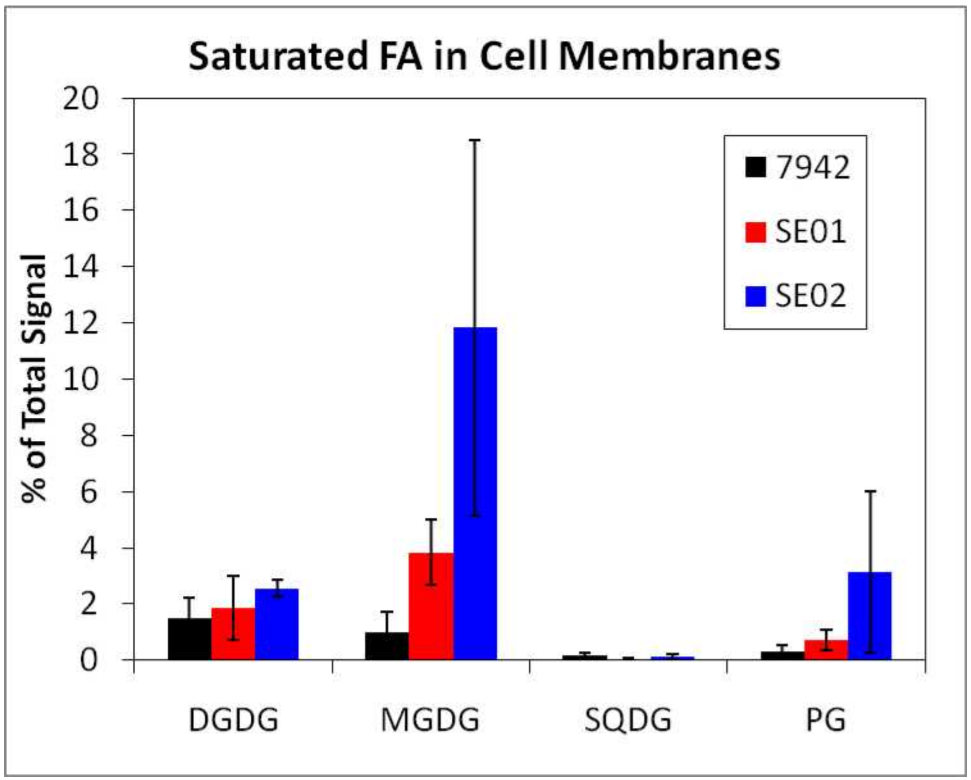
7942: Wild type

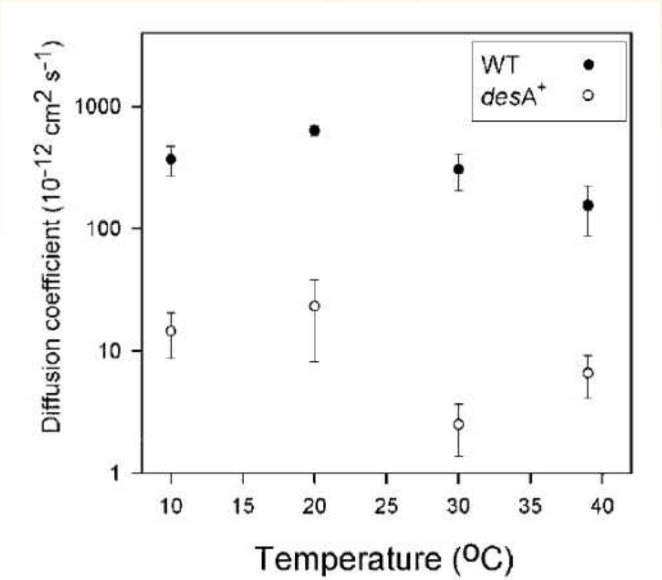
SE01: Knockout of acyl-ACP synthetase

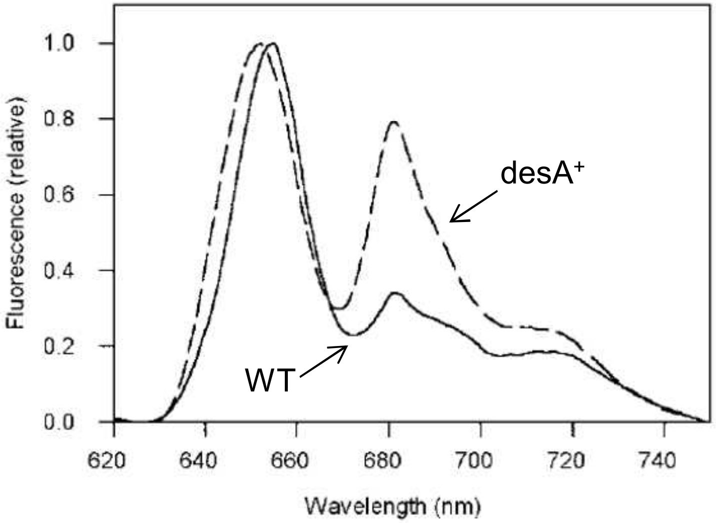
SE02: Knockout of acyl-ACP synthetase; expression of thioesterase (‘*tesA* from *E. coli*)

Changes in FA Composition of Membranes









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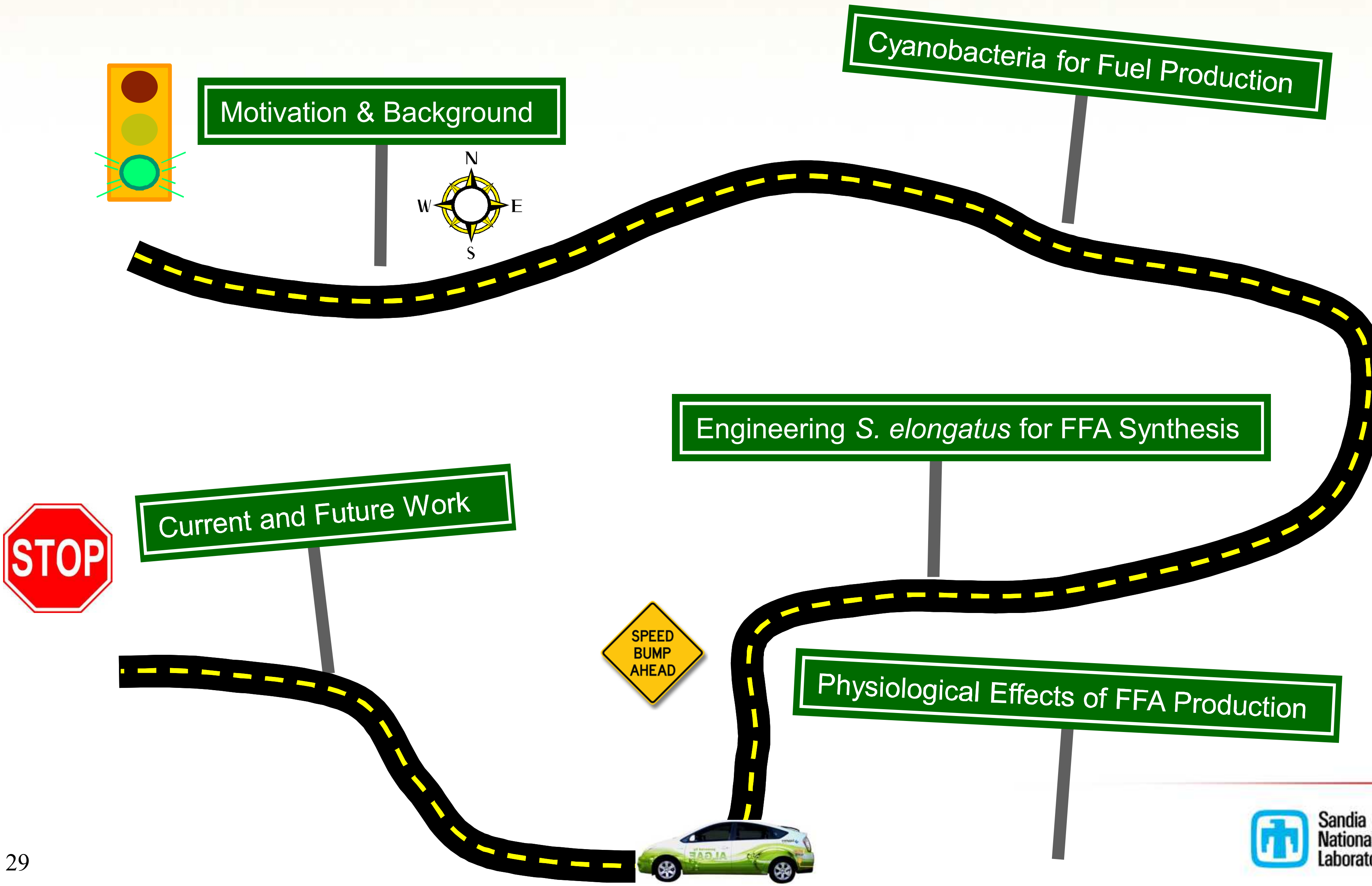
3. Sarcina M, Tobin MJ, Mullineaux CW. (2001) *J. Biol. Chem.* 276(50): 46830-4.

Roadmap to Cyanobacteria-Based Fuels



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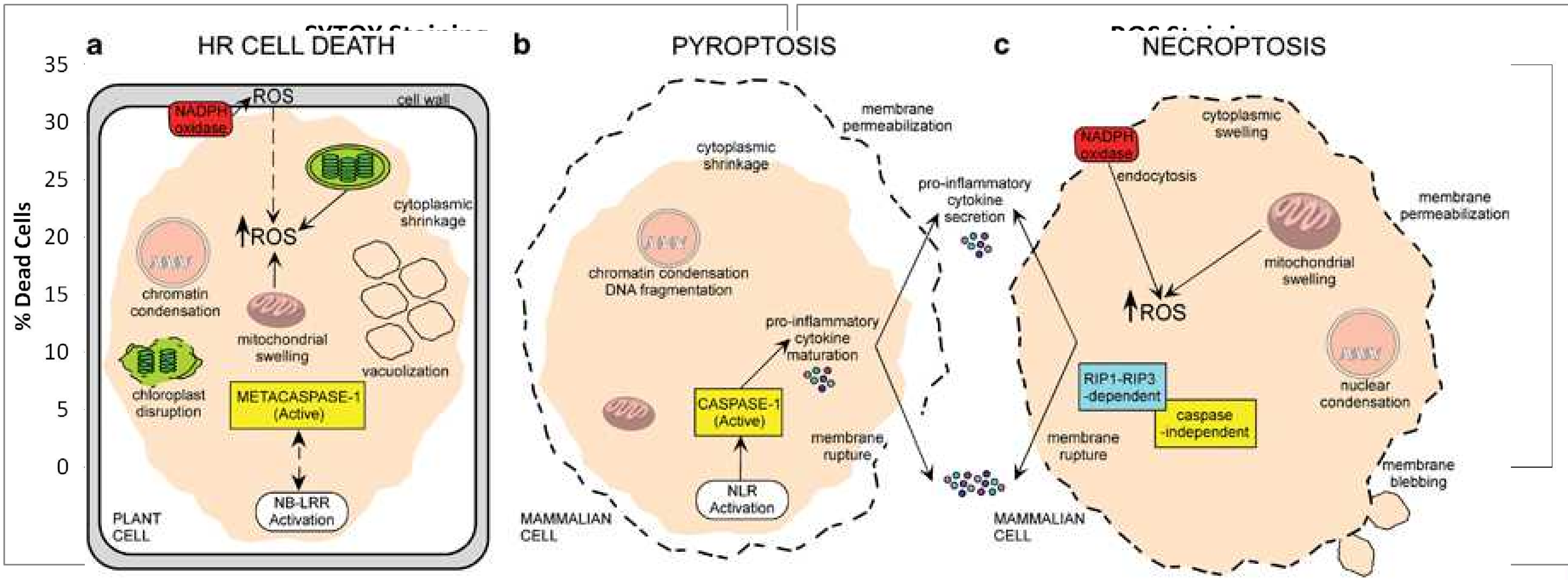
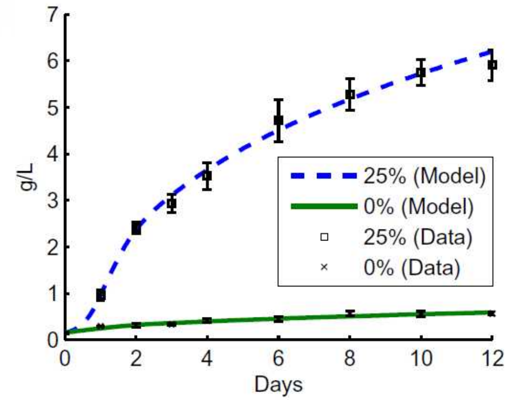
Cell Death and Reactive Oxygen Species



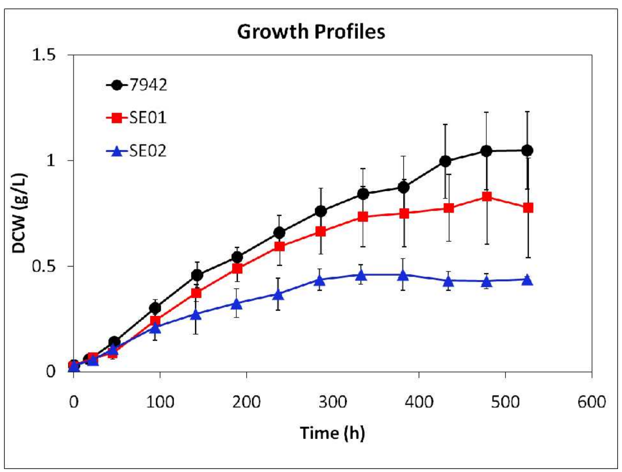
Coll NS, Epple P, Dangl JL. (2011) *Cell Death & Differentiation*. 18: 1247-56.

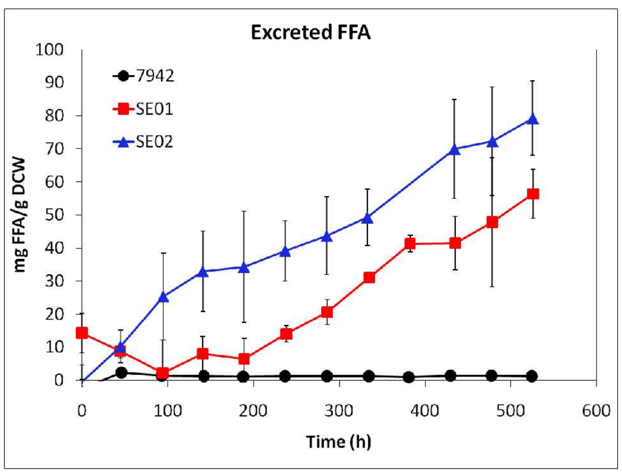
RNA-seq

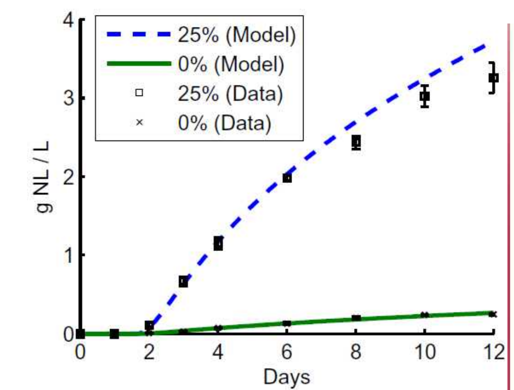
- Is FFA-induced cell death regulated by a programmed cell death mechanism?
- Are there any natural defense mechanisms to cope with increased ROS?
- Are there any active transporters involved in the export of FFA?
- FFA production is highest in the stationary phase; how is this regulated?
  - Possible correlation with high lipid production in eukaryotic algae under stress



(a) Total dry weight





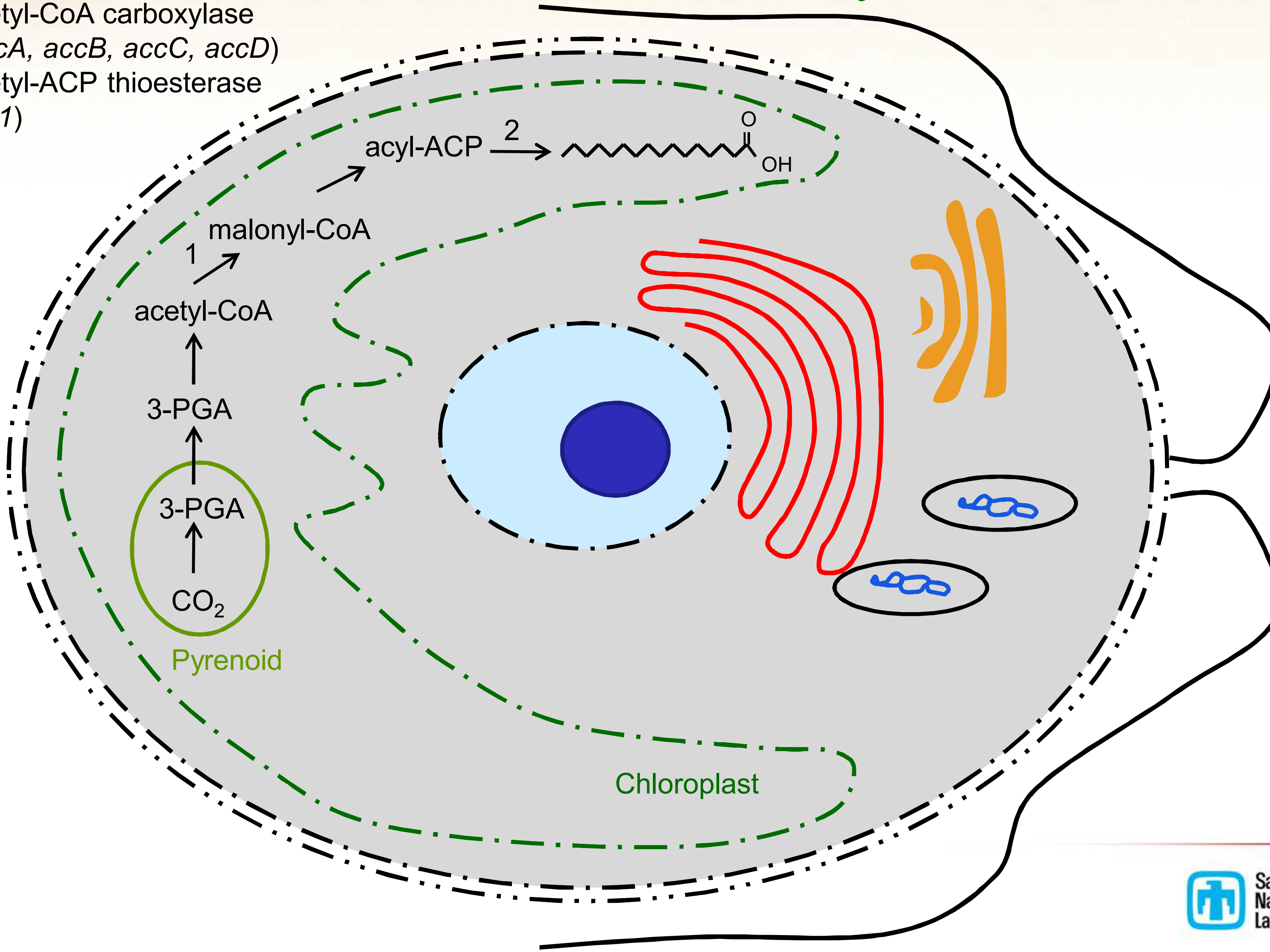


(a) NL density

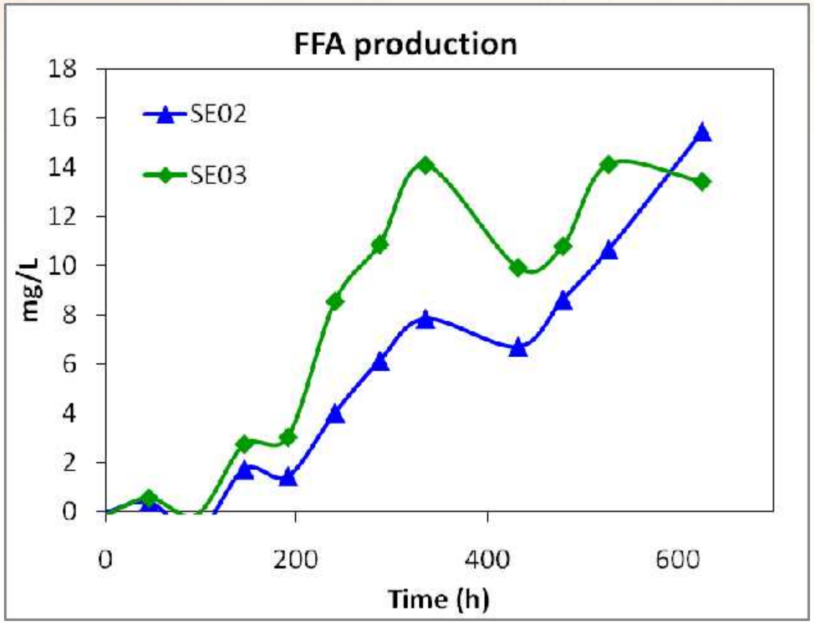
Packer A, Li Y, Andersen T, Hu Q, Kuang Y, Sommerfeld M. (2011) *Bioresource Technology*. 102: 111-7.

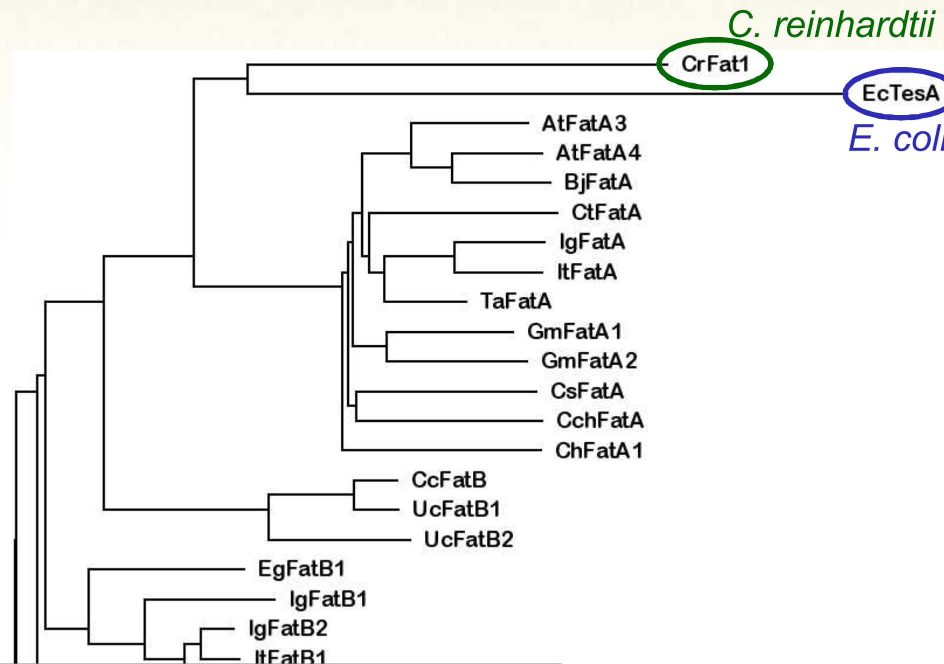
FFA Biosynthesis Genes from the Green Alga: *Chlamydomonas reinhardtii*

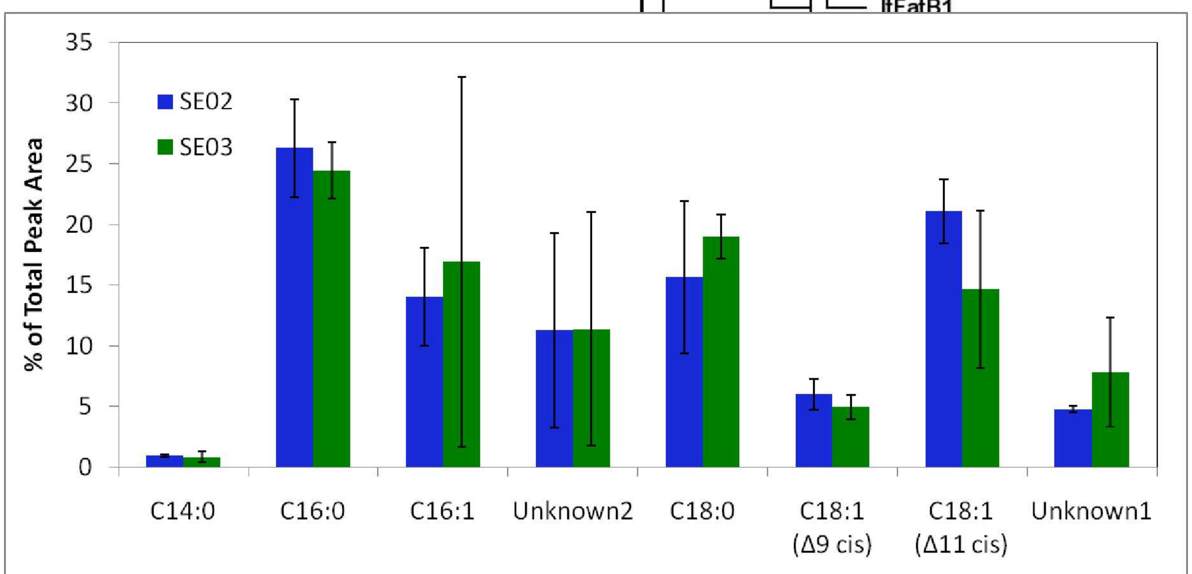
1: acetyl-CoA carboxylase
(*accA, accB, accC, accD*)
2: acetyl-ACP thioesterase
(*fat1*)



Fat1 from *Chlamydomonas reinhardtii*



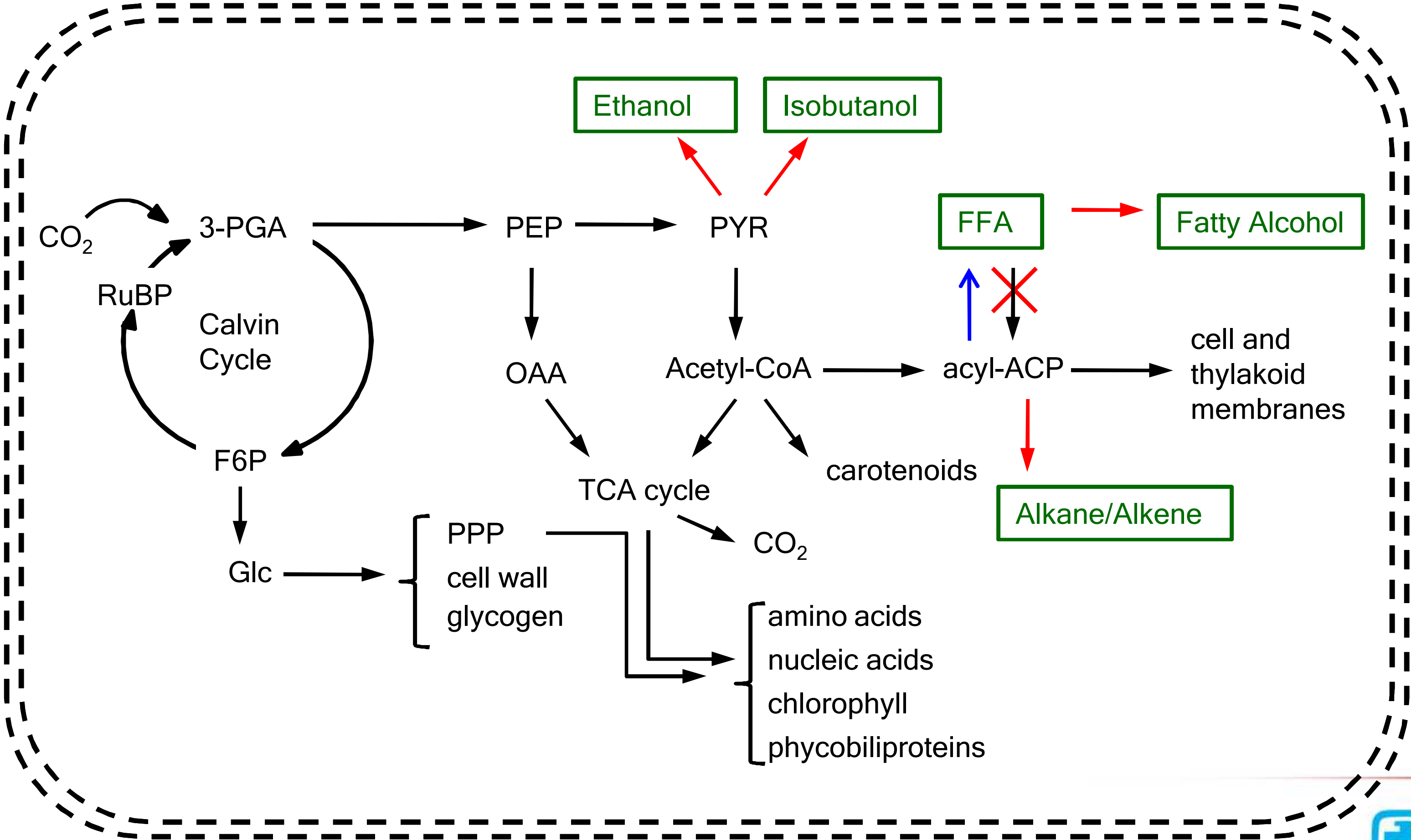




Cyanobacteria: Toxicity of Biofuels

Cyanobacterial strains:

- *Synechococcus elongatus* PCC 7942
- *Synechocystis* sp. PCC 6803
- *Synechococcus* sp. PCC 7002



Conclusions and Future Work

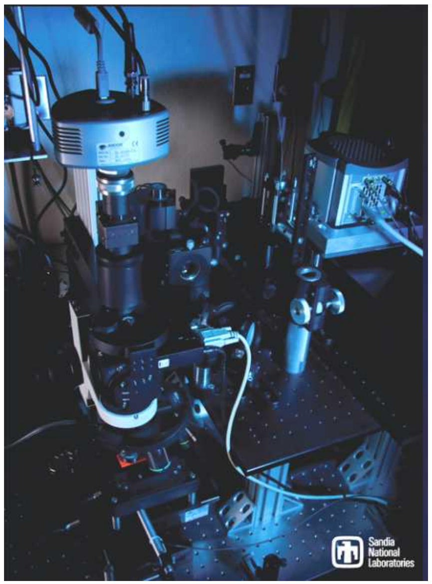
Conclusions

- Engineered cyanobacteria are promising candidates for biofuel production
- Characterization and an understanding of the effects of genetic manipulation are essential for strain development
- Saturated fatty acids are advantageous for biodiesel production
    - SFA does not affect cell growth, pigments, or photosynthesis
    - SFA have greater oxidative stability

Future work

- Identify an acyl-ACP thioesterase for the preferential release of SFA
- Investigate the physiological effects of other biodiesel precursors (i.e. long chain alkanes or alcohols)
- Additional metabolic engineering to enhance production of biodiesel feedstock

Acknowledgements



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Adaptive Evolution: FFA Tolerance