DOE Bioenergy Technologies Office (BETO)
2021 Project Peer Review

Sandia National Laboratories

REAL HYPE: Respiration Engineering of Algal Losses for High Yields and Productivity Enhancement

March 9, 2021

Advanced Algal Systems

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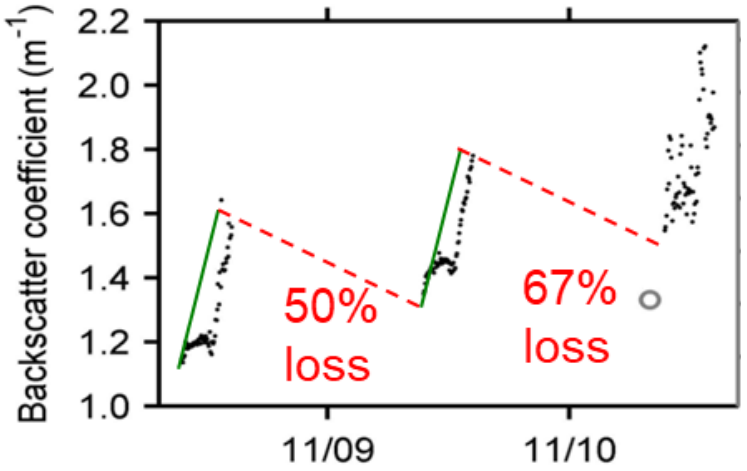
Project Overview

- Current algal biofuel productivities cannot support economic algal fuel production[1]
- During the day, algae store excess carbon as carbohydrates or lipids. At night, stored carbon is degraded to support energy production via dark respiration → loss of CO_2 (dark loss)
- Dark loss for *Microchloropsis* species can account for > 60% loss of biomass on a daily basis.
- Objectives (SEED project):
  - Year 1 (FY20): Use CRISPR-Cas9 tools to knockout 5 gene targets associated with dark loss to improve biomass productivity by 10% in *Microchloropsis*
  - Year 2 (FY21): Develop CRISPR interference tools and test combinatorial knockdowns of gene targets associated with dark loss to improve biomass productivity by 20% in *Microchloropsis*
- *Microchloropsis gaditana* CCMP526 has draft genome sequence and CRISPR-Cas9 genome editing tools were previously developed at Sandia (LDRD funding)


Image: MicroBioEngineering

Growth of *M. salina* in outdoor mini-raceway pond (ATP[3])



1. U.S. Department of Energy (2020), Bioenergy Technologies Office 2019 R&D State of Technology, DOE/EE-208.

1-Management

- Project management (seed project, SNL)
  - Biweekly team meetings
  - Twice quarterly meetings with BETO technology manager and project monitor
  - Quarterly reports
  - Publication and presentation of results
- Team members and responsibilities:

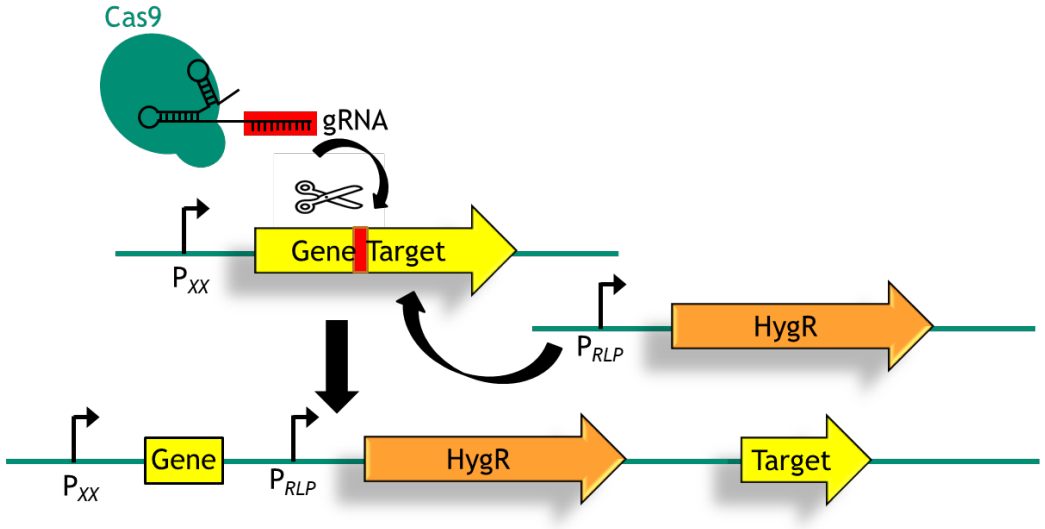
Name	Position	Responsibilities
Anne Ruffing	Principal Investigator	Project management, experimental design, experiments, and data analysis
AnaLisa Ortiz	Undergraduate student intern	Experiments and data analysis
Tessily Hogancamp	Postdoctoral Appointee	Evening OD measurements for growth characterization (COVID evening shift)
TBD, offer extended Jan 2021 (hiring delay due to COVID)	Postdoctoral Appointee	Experimental design, experiments, and data analysis

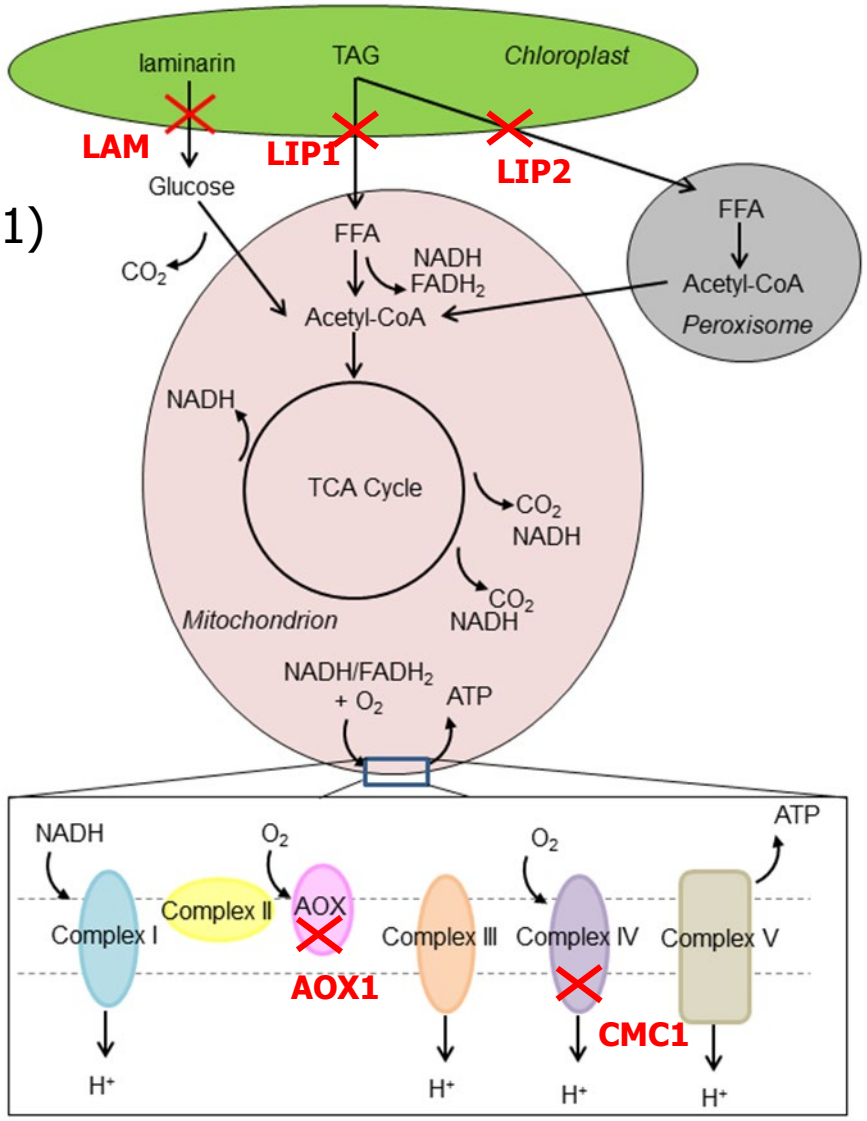
1-Management

Risk Description	Response Plan	Severity	Status
Targets for gene knockout may be essential	Any essential genes that cannot be knocked out will be knocked down using CRISPRi	Moderate	Not realized
Construction of dCas12a gene may fail (large size, high A/T content, and potential toxicity in E. coli cloning strains)	We will pursue multiple strategies for construction of dCas12a. We will attempt to order a codon-optimized dCas12a from GenScript. We already have a plasmid with Cas12a from Francisella novicida which will be mutated to construct the 'dead' version. As a backup plan, we can use dCas9, which will require a larger single guide RNA construct for multiplexed targeting.	Moderate	Not realized
CRISPRi may fail due to gene silencing or other unknown regulatory mechanisms	We will optimize promoters for dCas12a and gRNA expression and consider other factors to improve expression, such as the inclusion of native introns. If gene knockdown with dCas12a cannot be achieved, dCas9 will be pursued as a backup strategy.	Moderate	In progress

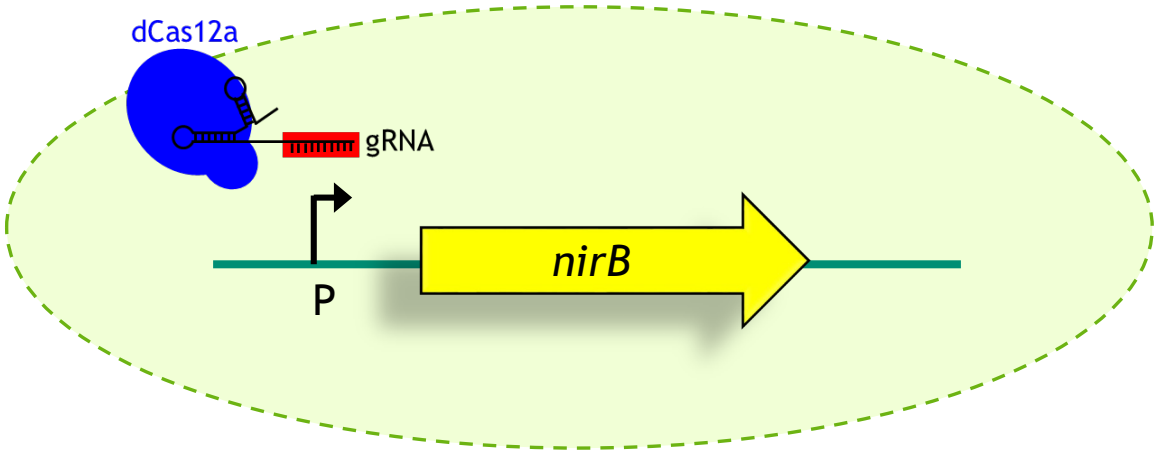
2-Approach
CRISPR Editing for Single Gene Knockouts

- Five gene targets selected for knockout to reduce dark loss
  - Prevent degradation of storage carbohydrates and lipids (LAM, LIP1, LIP2)
  - Reduce flux through dark respiration pathway (AOX1 and CMC1)
- Design guide RNAs (gRNAs) in transient expression plasmid to target each of the 5 genes
- Transform the gRNA plasmid and selection cassette into Cas9-expressing *M. gaditana*
- Confirm gene knockout with PCR screening



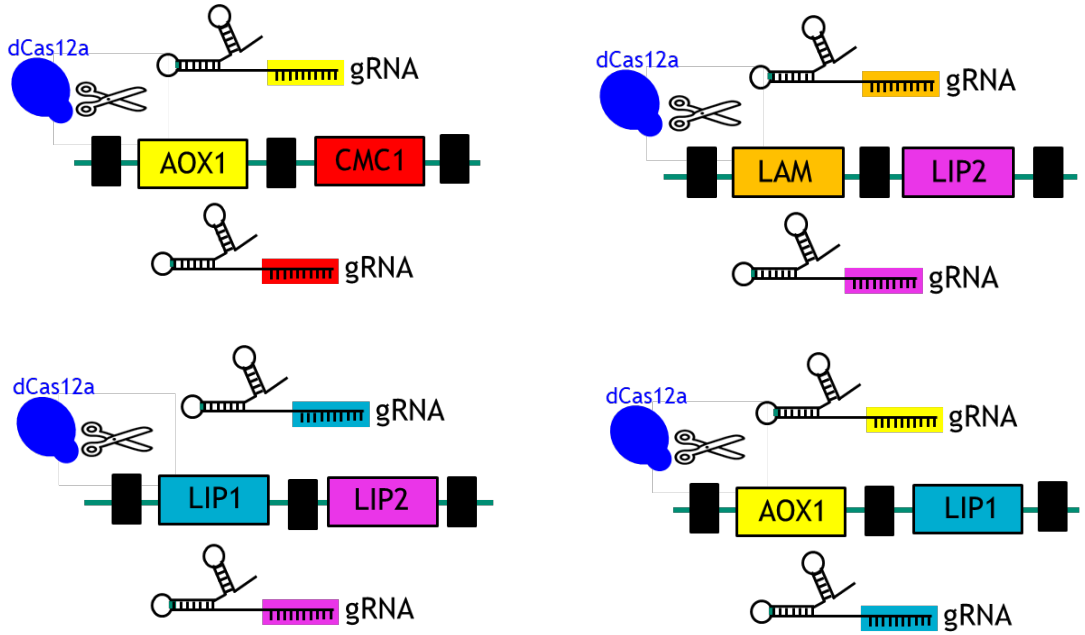


2-Approach
CRISPR Interference for Multi-Target Gene Knockdowns



Demonstrate CRISPR interference (CRISPRi) targeting nitrate reductase (*nirB*)

- Construct plasmid with codon-optimized dCas12a variants (dCas12a, dCas12a-KRAB, and dCas12a-3xSRDX) and gRNA targeting *nirB*
- Randomly integrate into the genome of *M. gaditana*
- Measure changes in growth on media with nitrate as the sole nitrogen source

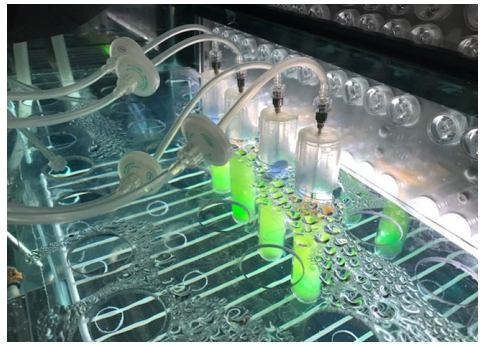


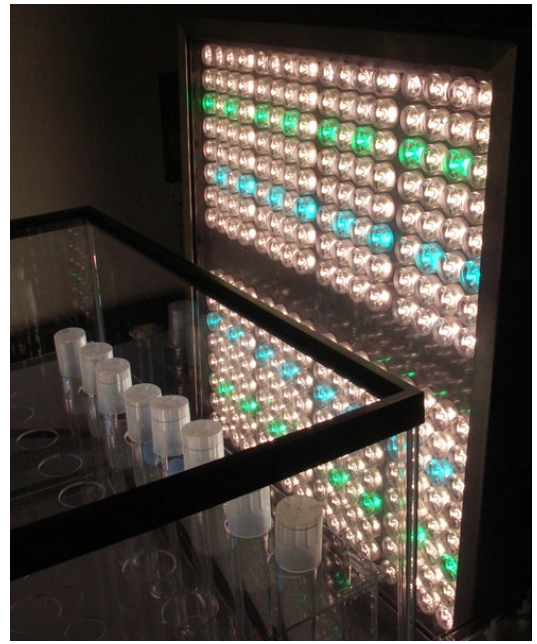
Multiplexed CRISPRi to reduce dark loss

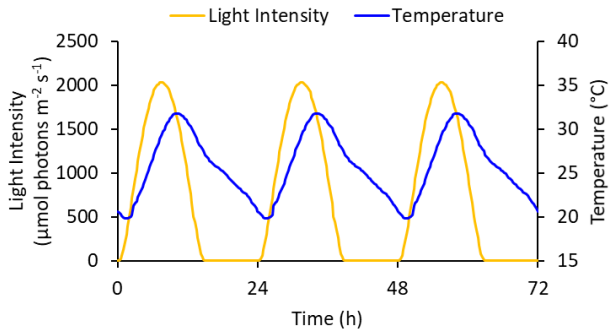
- Design gRNA arrays targeting 2-gene combinations of LAM, LIP1, LIP2, AOX1, CMC1
- Randomly integrate into genome of *M. gaditana*
- Measure changes in cell growth (OD) and lipid content (bodipy staining/flow cytometry)

2-Approach
Strain Characterization

- Grow knockout and knockdown mutants in 50 mL culture tubes with simulated light and temperature conditions
  - Mimic outdoor conditions from outdoor raceway experiment at ATP^3 in June 2014
  - Medium: F2N-NO_3
  - Aeration: continuous air bubbling
- Measurements:







Parameter	Method	Frequency
Growth	Optical density at 750 nm	Twice daily (lights on and lights off)
Growth	Ash free dry weight (AFDW)	End of cultivation period
Lipid content	Bodipy staining and flow cytometry	Every 2 days for LIP mutants, end of cultivation period for other strains
Carbohydrate content	Acid-phenol absorbance method	End of cultivation period

3-Impact

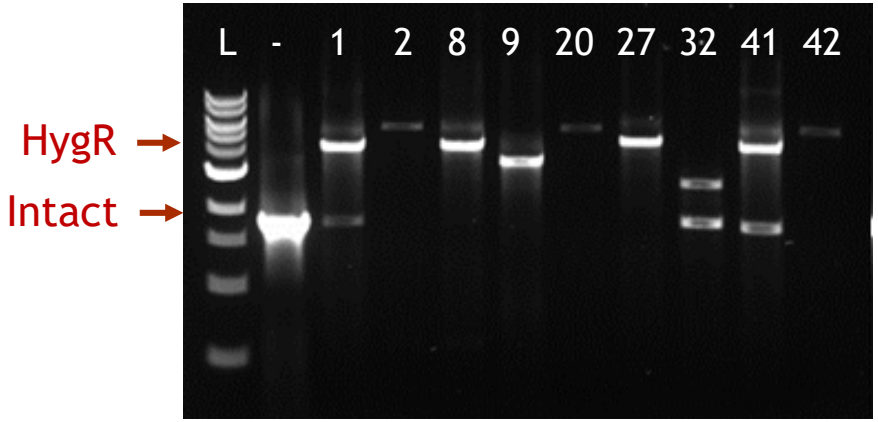
1. Algal biomass productivity
   - *M. gaditana* strain with 20% improvement in biomass productivity will be a significant improvement towards the 2030 target productivity of 25 g/m^3/day
   - Additional efforts may be necessary for outdoor use:
     - Approval for GMOs or mutation methods that avoid recombinant proteins
     - Scale-up testing → 100 L mini-raceway ponds at SNL greenhouse
   - Genetic targets may also improve biomass productivities in other algal strains by reducing dark loss
2. Algal genetic toolbox development
   - CRISPRi tools may be used in future studies to target any gene or combination of genes
   - Enables future work with high-throughput gRNA library screening for a wide range of applications
   - CRISPRi tools may be translated into other algal species

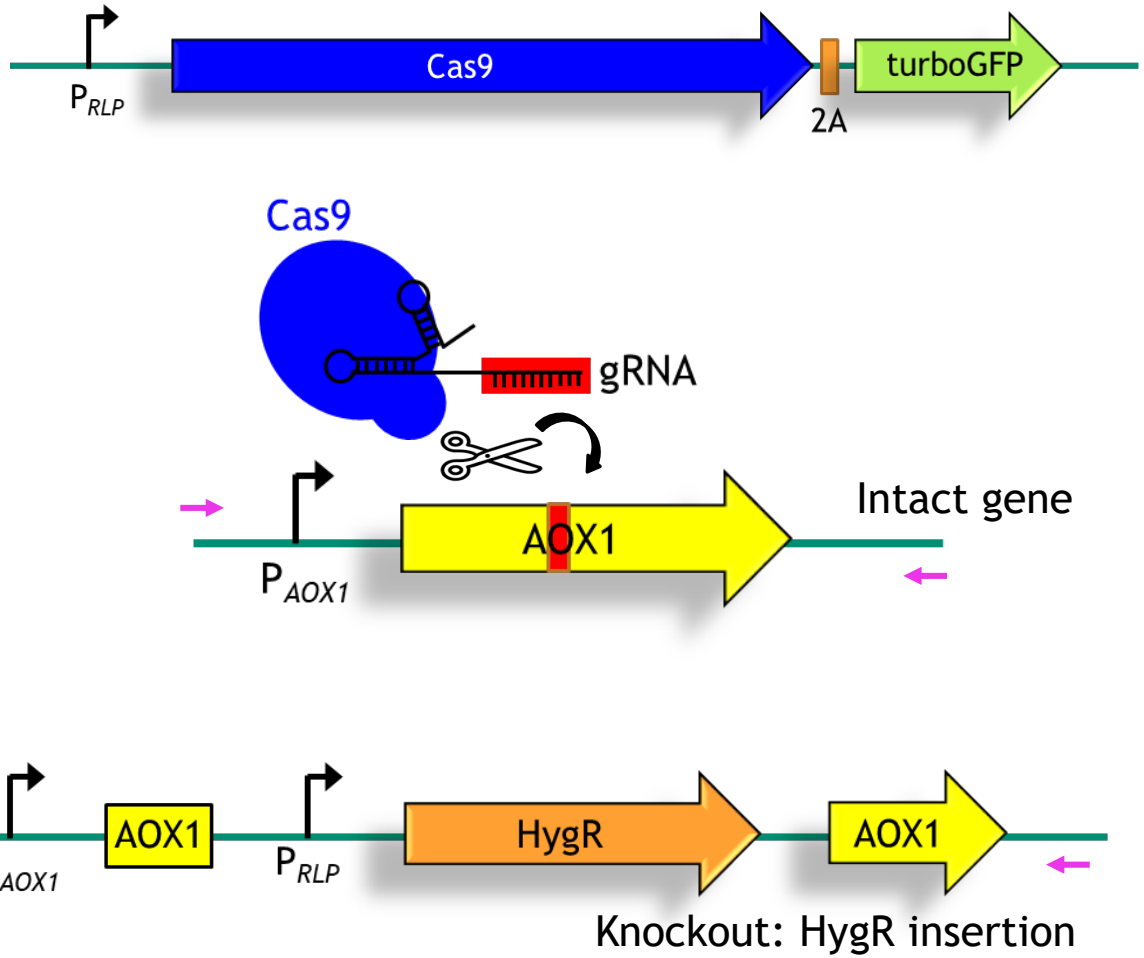
- Dissemination of results
  - Publish two peer-reviewed articles
  - Present results at conferences and seminars
  - Strains will be made available by request
  - Plasmid constructs for gRNA and CRISPRi will be available through Addgene (pending SNL legal approval) or by request

4-Progress and Outcomes
Single Gene Knockouts (Task 1)

- Established CRISPR-Cas9 editing in *M. gaditana* during previous project (LDRD-funded)
- Constructed gRNAs for all 5 gene targets and transformed into MG-Cas9^{+} strain along with selection marker
- Successful knockout of all 5 gene targets
- Editing efficiencies in resistant colonies = 3 – 64%

AOX1-1, PCR screen



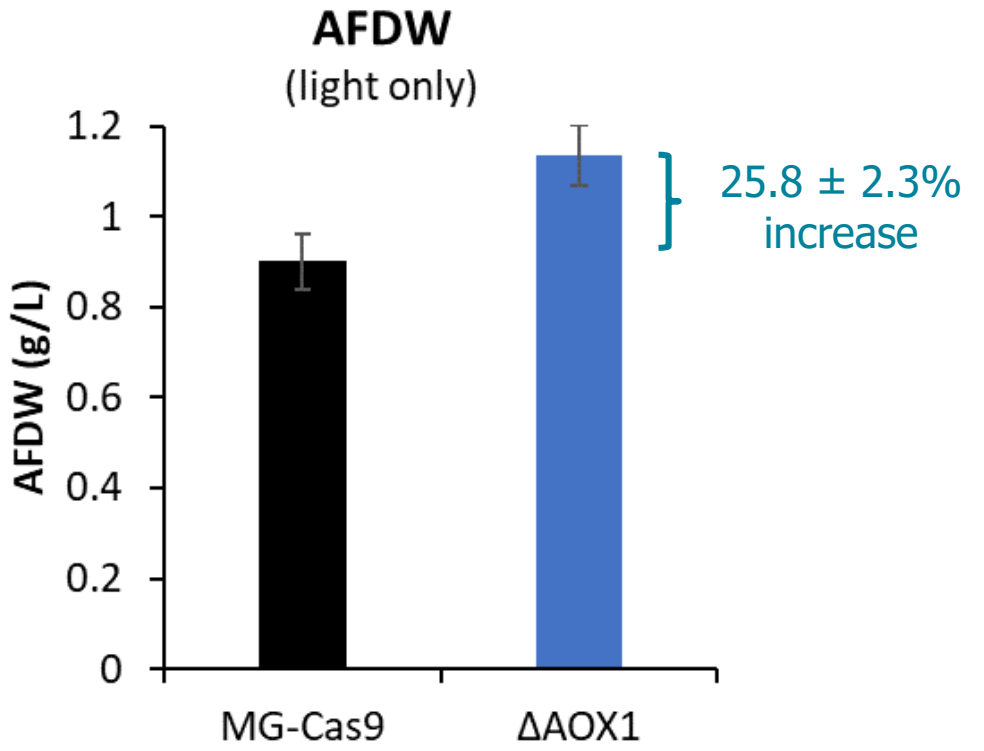


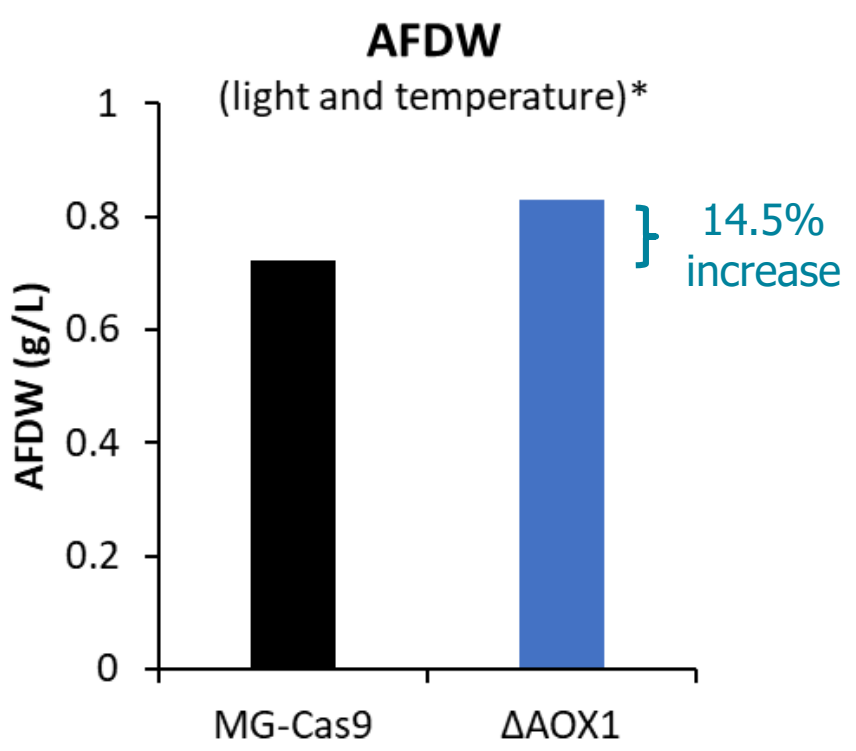
Successfully completed Go/No-Go milestone.

4-Progress and Outcomes

ΔAOX1 Improves Biomass Productivity

*Additional experimental replicates are in progress.

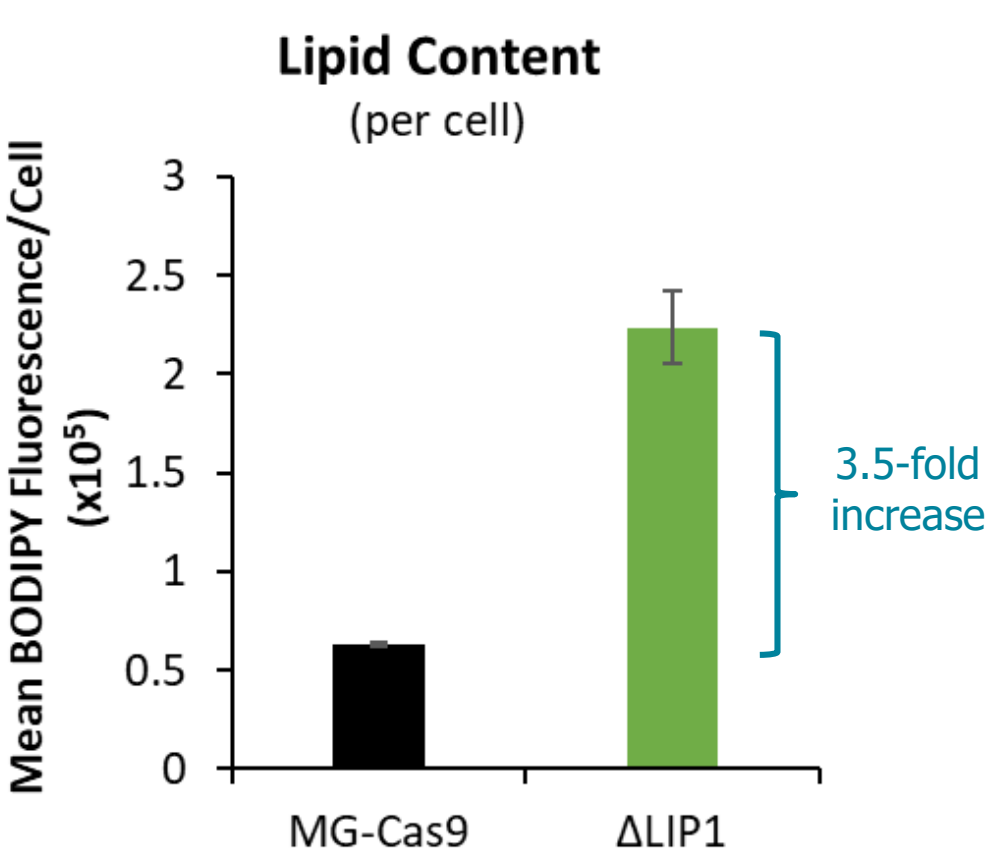


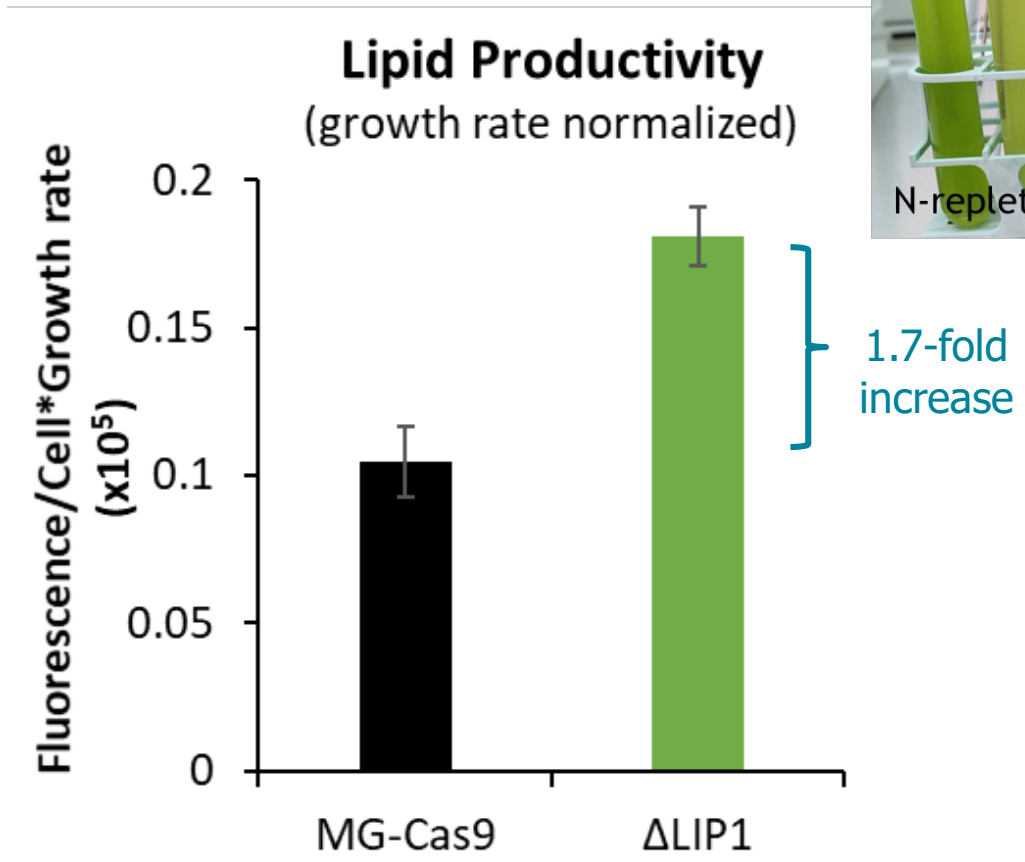


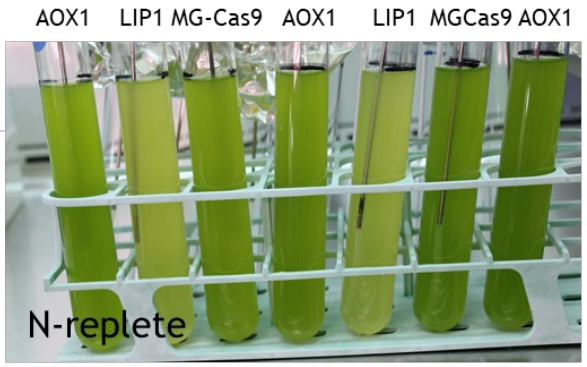
- Two 3-week cultivation experiments showed improved AFDW for ΔAOX1 relative to parental strain (MG-Cas9)

Fulfills FY20 Q4 annual milestone (10% improvement in AFDW)

4-Progress and Outcomes
ΔLIP1 Improves Lipid Content







- 18-day cultivation experiment showed increased lipid content and productivity in ΔLIP1 relative to parental strain (MG-Cas9) **without nitrogen depletion**

5-Summary

- Achieved ‘Go’ criteria for project Go/No-Go milestone on constructing five gene knockout strains in *M. gaditana*
- In 50 mL culture tubes, ΔAOX1 strain demonstrated 14.5% increase in AFDW under 20 days of simulated summer light and temperature conditions
- In 50 mL culture tubes, ΔLIP1 strain had 1.7-fold increase in lipid productivity without nitrogen deprivation
- Preliminary CRISPRi results are inconclusive; troubleshooting in progress

Future Work

- Scale-up testing of ΔAOX1 and ΔLIP1 strains in greenhouse outdoor 100 L mini-raceway ponds (approved for GMO testing)
- Transcriptomic, proteomic, and metabolomic analysis of ΔAOX1 and ΔLIP1 to understand growth and lipid phenotypes

Acknowledgements

Sandia National Laboratories (SNL)

Staff members

- **Anne Ruffing**
- Todd Lane
- Pamela Lane

Post-docs

- **Tessily Hogancamp**
- Jordan McEwen (DTRA)

Undergraduate Interns

- **AnaLisa Ortiz**
- Stephanie Kolker (RPI, PhD candidate)
- Ian Lubkin
- Lucas Strickland (UCLA, PhD candidate)

Bioenergy Technologies Office

- Technical Manager: Devinn Lambert
- Project Monitors: Philip Lee and Elizabeth Burrows

Funding Sources

U.S. DEPARTMENT OF ENERGY | Energy Efficiency & Renewable Energy

BIOENERGY TECHNOLOGIES OFFICE

LDRD LABORATORY DIRECTED RESEARCH & DEVELOPMENT — WHERE INNOVATION BEGINS

Quad Chart (AOP)

Timeline

Project start date: 10/01/2019

Project end date: 09/30/2021

	FY20	Active Project
DOE Funding	$175k	$400k

Project Partners

N/A – Seed project

Barriers addressed

19AFt-B: Sustainable Algae Production

19Aft-C: Biomass Genetics and Development

Project Goal

The goal of this project is to develop and apply CRISPR genetic engineering tools to reduce carbon dioxide lost at night during dark respiration (dark loss), leading to improved biomass productivities in the alga *Microchloropsis gaditana*.

End of Project Milestone

Small-scale demonstration of 20% improvement in growth of *M. gaditana* GM strains relative to the wildtype under simulated light and temperature conditions

Funding Mechanism

FY20 AOP Lab Call

Responses to Previous Reviewers' Comments

- Not applicable – this is the first peer review for REAL HYPE

Presentations

- Invited talks:
  - Ruffing AM. *Bioengineering and National Security: Engineering Photosynthetic Microorganisms for Energy Security and Developing Biosensors for Nuclear Security.* Davidson School of Chemical Engineering Graduate Seminar Series, Purdue University. October 2019. Lafayette, IN.
  - Ruffing AM. *Bioengineering and National Security: Engineering Photosynthetic Microorganisms for Energy Security and Developing Biosensors for Nuclear Security.* Bioscience Seminar, Los Alamos National Laboratory. January 2020. Los Alamos, NM.
- Oral Presentations:
  - Ortiz A. *REAL HYPE: Respiration Engineering of Algal Losses for High Yield and Productivity Enhancement.* Bioscience Summer Intern Symposium, Sandia National Laboratories, August 5, 2020. (virtual)
  - Ruffing AM. *Challenges in Developing a Syn-Bio Toolkit for Microchloropsis gaditana*. Algae Rules of Life Workshop (BETO/NSF), Virtual Summer Symposium, lightening talk, September 25, 2020.
  - Ruffing AM. *CRISPR-mediated engineering of photoautotrophs to improve biomass productivity.* 11th International Conference on Biomolecular Engineering (ICBE), January 9, 2021. (virtual)