DOE Bioenergy Technologies Office (BETO) 2021
Project Peer Review

PROGRESS TOWARDS A NEW MODEL CHEMOLITHOAUTOTROPHIC HOST

March 15, 2021
AGILE Biofoundry Consortium

Dan Robertson
Kiverdi

Project Overview

- *Cupriavidus necator* has the potential to be a model engineering host for direct bioconversion of CO_2 to value-added products
  - Performs autotrophic, *e.g.*, knallgas, fermentations on CO_2 + H_2 + O_2, or heterotrophic fermentation on sugars or glycerol
  - Grows rapidly to high density (OD ~200) on CO_2; H_2O is the only byproduct
  - Energetically superior metabolism for product synthesis; High productivity (>2g/L/hr) and yield on CO_2
  - History of use for industrial production of a natural polymer, PHB
- Kiverdi has developed strains, reactors, fermentations and processes and demonstrated technoeconomic and life cycle advantages
- LBNL and ORNL have experience engineering non-model engineering hosts
- NREL brings experience with development of gene editing tools

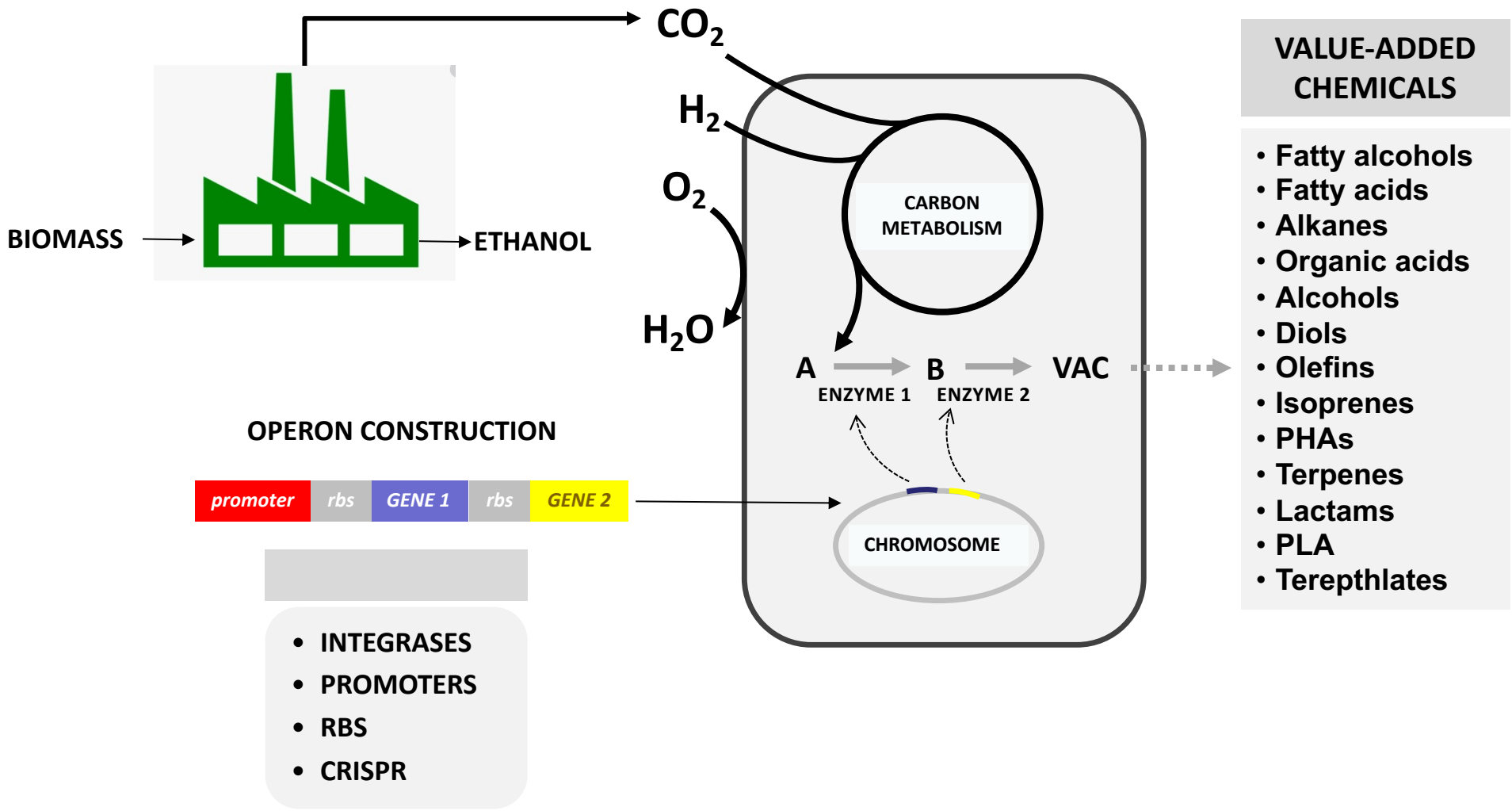
Challenges

- Genetic tools for *C necator* are limited, making metabolic engineering slow and laborious
- DNA transformation efficiencies of *C. necator* are poor
- There is currently no general method for chromosomal integration of pathways into *C. necator*
- Control elements for gene expression are limited and not well-developed
- It is difficult to inactivate multiple genes in *C. necator*
- Reaching productivity goals of non-native products in *C. necator* has been challenging

Project Goals

- Develop a multi-level metabolic engineering toolbox for *C necator* comprised of:
  - An efficient DNA transformation and chromosome integration system for *C necator* (ORNL)
  - A *C necator* CRISPR gene editing system (NREL)
  - Libraries of genetic control elements with broad ranges of amplification; ribosome binding sites (RBS), promoters (ORNL)
- Construct and express the genes of an engineered metabolic pathway for the C_{12} fatty alcohol, dodecanol (Kiverdi/LBNL)
- Demonstrate dodecanol production from $CO_2/H_2/O_2$ in lab bioreactor (Kiverdi)
- Using the toolbox, fine tune pathway gene expression, delete competing pathways, modulate metabolic regulation for highest dodecanol yield (All)

Knallgas Engineering Chassis



Heilmeier Catechism

1. What are we trying to do?

- Deploy a platform biotechnology that leverages the distinct advantages of knallgas fermentation for productivity and sustainability.
- Develop a toolbox enabling creation of a model *C necator* chassis for engineering the production of a broad range of commodity and specialty chemicals.
- Demonstrate a knallgas process for dodecanol synthesis from CO_2 to replace non-sustainable production from palm oil.

2. How is it done today and what are the limits?

- Unlike knallgas, most heterotrophic fermentations produce CO_2 or other byproducts that divert carbon and energy, and impact productivity and yield.
- Chemicals have been produced in metabolically engineered *C necator* though not at industrially relevant yields.

3. Why is it important?

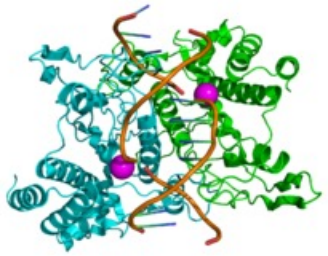
- The ability to fine tune engineered pathways in an organism with an industrially beneficial phenotype will enable high productivity in sustainable CO_2-consuming processes

4. What are the risks?

- The current cost of H_2 is the greatest cost of goods (COGS) sensitivity for scaled dodecanol production. It is forecast that by 2025 green hydrogen at a projected $1/kg will match the long-term breakeven price in Europe of natural gas ($7.5/mmBTU), achieving fossil fuel parity
- Productivity has a large effect on the predicted internal rate of return (IRR) of the dodecanol process. The tools developed in the project will enable fine tuning of metabolism and direct carbon to dodecanol

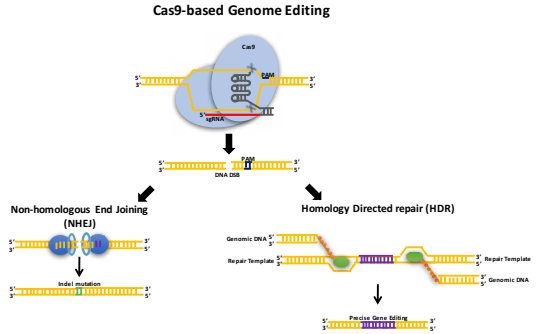
Project Structure

Altering *C. necator* restriction modification system



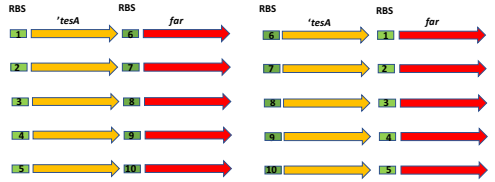
Adam Guss

Developing Cas9 gene editing



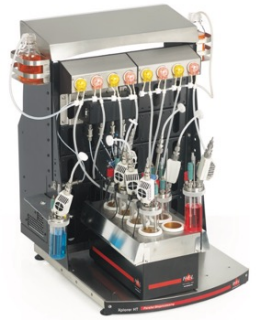
Carrie Eckert

RBSs for 1-dodecanol production



Steve Singer
Kenneth Zahn

Bioreactor experiments



John Reed
Kenneth Zahn

- Project manager is Steve Singer, LBNL
- Project tasks are managed by the institutional Project Leaders
- Tasks, timelines, milestones and deliverables are defined in the project plan
- Quarterly updates are conducted; focusing on progress to milestones, experimental and operational challenges and mitigations

Technical Strategy

- Improve *C. necator* transformation efficiency at least 10-fold (ORNL).
- Identify at least 1 functional CRISPR/Cas system for genome editing (NREL).
- Demonstrate simultaneous editing of at least 2 genes (NREL).
- CRISPR-based editing with pooled library > 4 mutations (NREL).
- Using RFP as a reporter, characterize expression strength in *C. necator* of at least 10 RBSs from a library with a wide range of calculated Translation Initiation Factors (TIFs) (LBNL, Kiverdi).
- Construct in *C. necator* a heterologous pathway for biosynthesis of 1-dodecanol from dodecanoic acid. Perform initial optimization of the pathway by:
  - Varying the RBSs in dodecanol pathway operon
  - Knocking out at least two acyl-CoA dehydrogenases (to disable β-oxidation) (LBNL/Kiverdi/ORNL).
- To facilitate commercialization, initiate optimization and scale-up of *C. necator* chemolithoautotrophic fermentation with H_2 and CO_2 (Kiverdi).

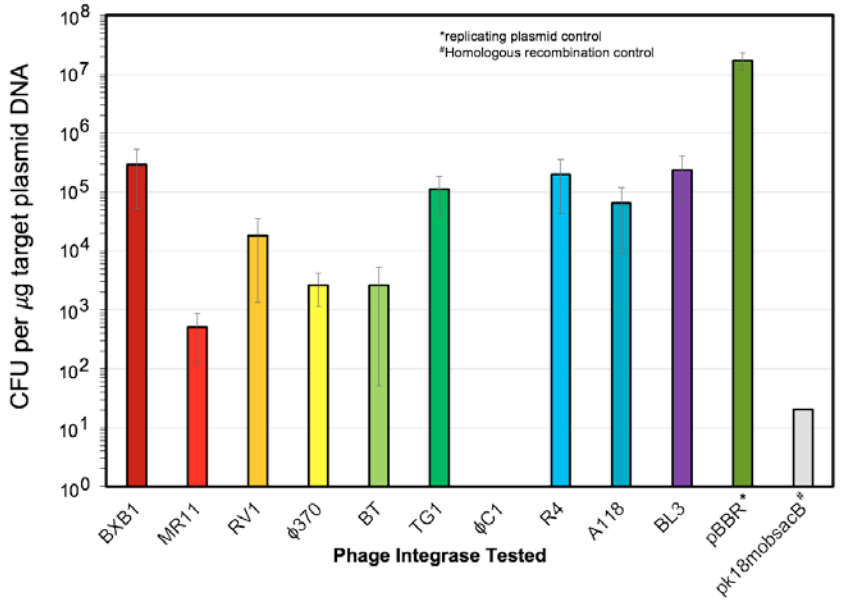
Impact

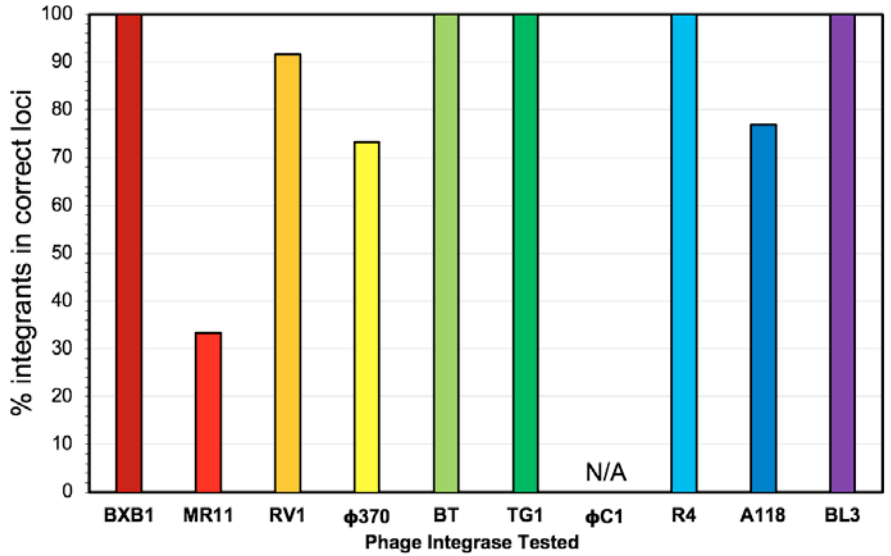
- Fatty alcohols are intermediates in the production of detergents, cosmetics and agricultural chemicals.
- The market is predicted to reach $10B/yr by 2023 with strongest growth in the surfactant sector.
- Fatty alcohols are currently produced either from triglycerides derived from palm, coconut and soy oils, or from fossil fuel-derived ethylene polymerization.
- The use of processing chemicals, deforestation and land diversion as well as waste generation by palm kernel oil production results in a large negative carbon footprint (estimated 5.27 CO_2e/kg dodecanol vs -2.4 CO_2e/kg dodecanol for knallgas.
- Replacing these molecules with renewable alternatives is essential to advance the US bioeconomy and to mitigate the climate effects of producing these molecules from palm oil and petroleum
- The project aligns with a strategic partnership between Kiverdi and SC Johnson, a world leader in surfactants

DNA Transformation

Task 1: **Method(s) for 10-fold improved transformation efficiency in *C. necator***

- *C. necator* electroporation efficiency was increased to 100 times greater than previously published protocols (*Biotechnol Biofuels* 2018 Jun 20;11:172)
- Recognition sites for 10 phage integrases were incorporated into *C. necator* chromosome at *H16_A0006* site
- High efficiency and insertional accuracy were achieved for several of the phage integrases

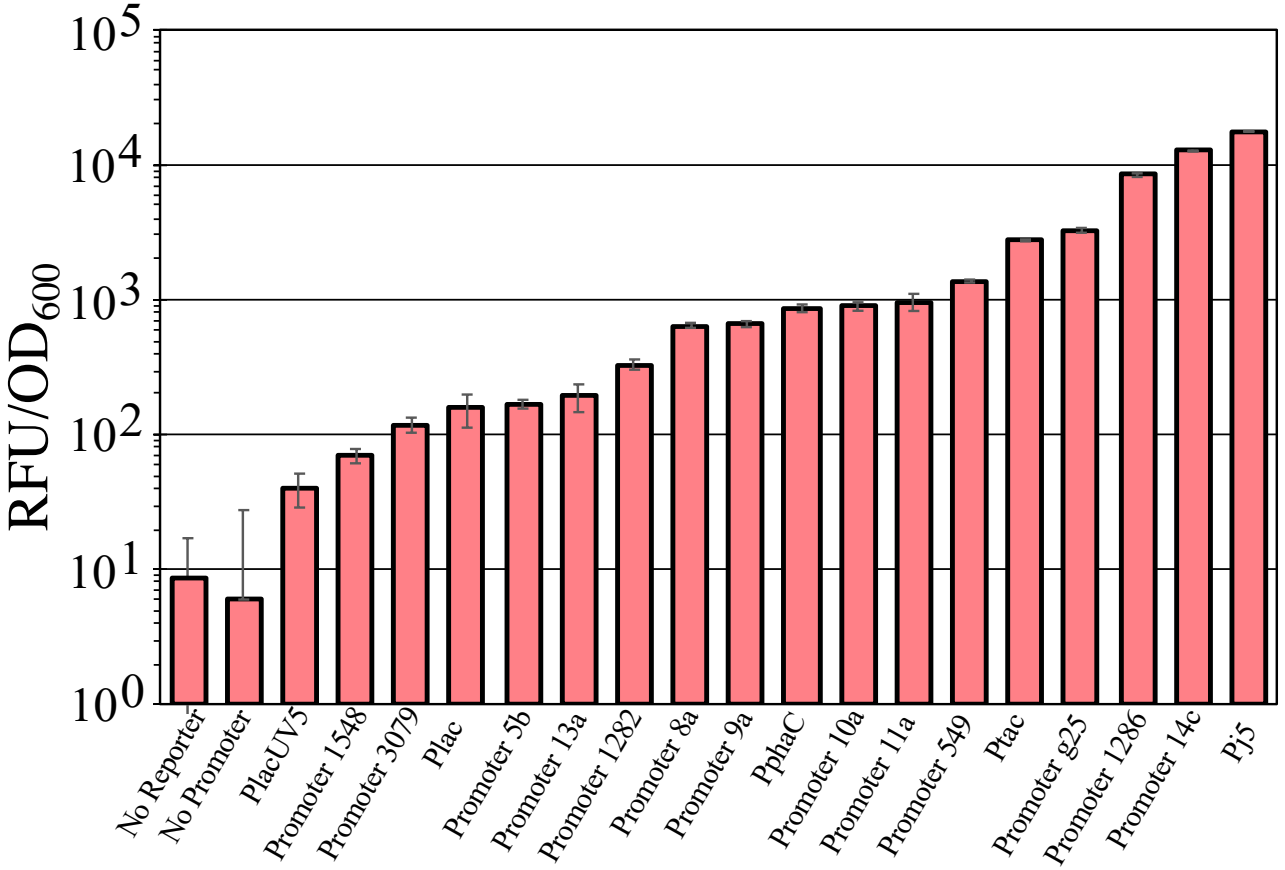




Promoter Library

Task 1: **Method(s) for 10-fold improved transformation efficiency in *C. necator***

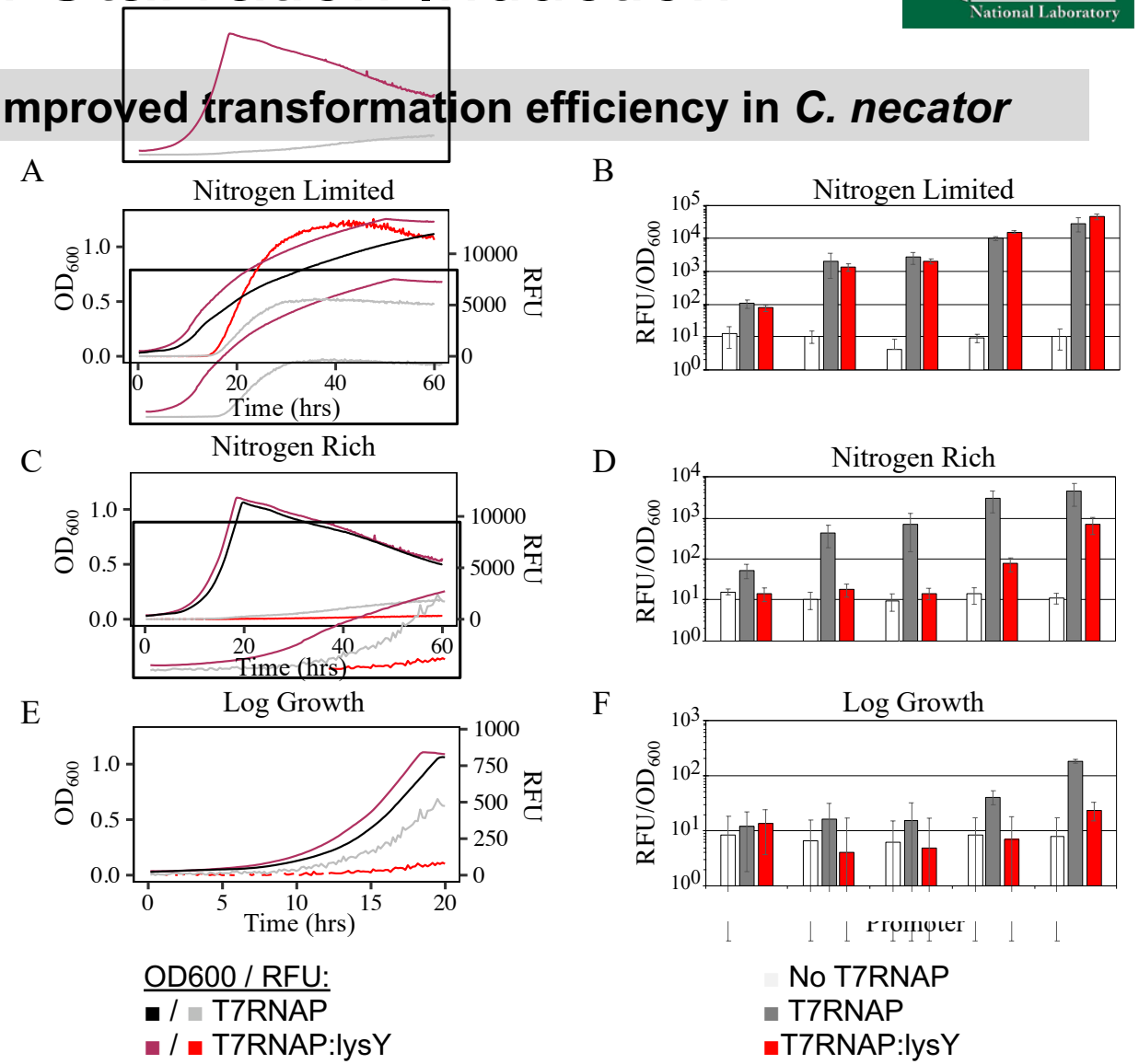
- Evaluated library of promoters expressing fluorescent protein from chromosome
- Promoters cover a 500-fold range of expression levels



Nitrogen Starvation Induction

Task 1: Method(s) for 10-fold improved transformation efficiency in *C. necator*

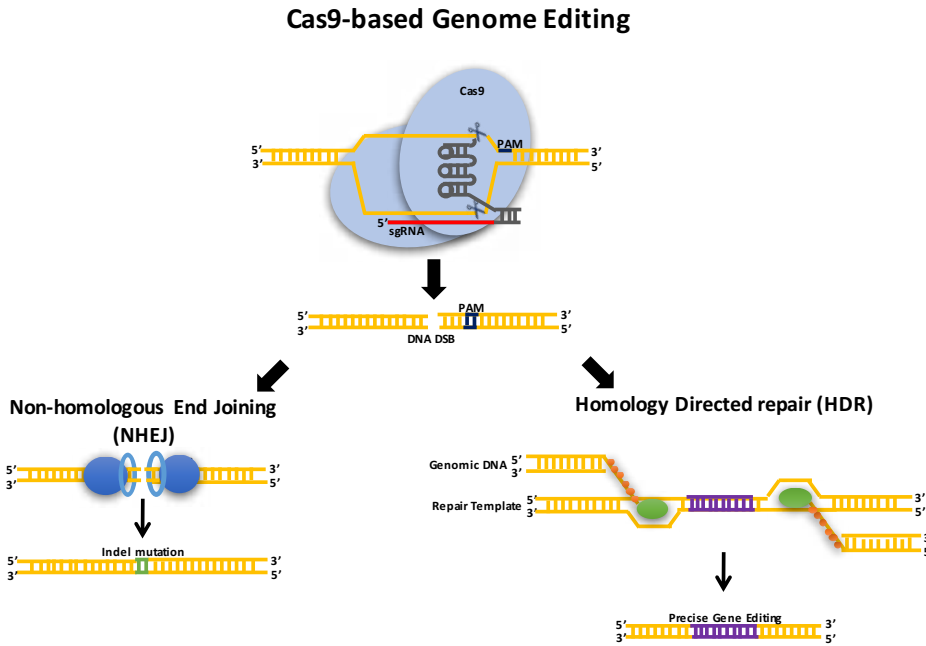
- Built inducible gene expression system using nitrogen starvation as the signal and T7 polymerase to amplify the signal
- Promoters span ~1000-fold range in expression upon N-starvation

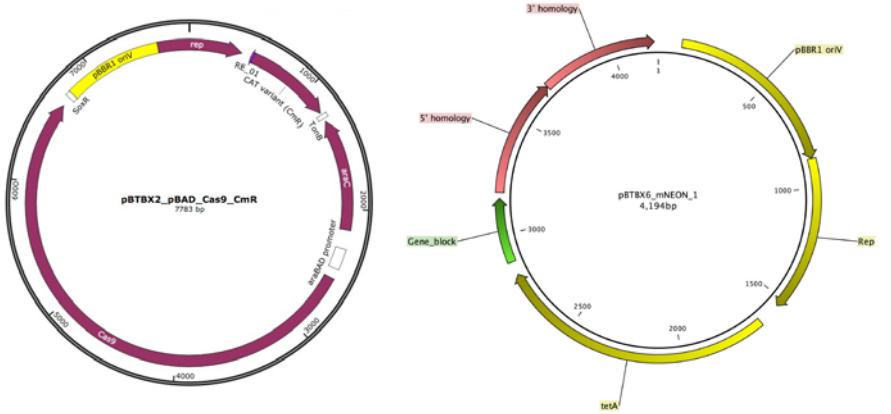


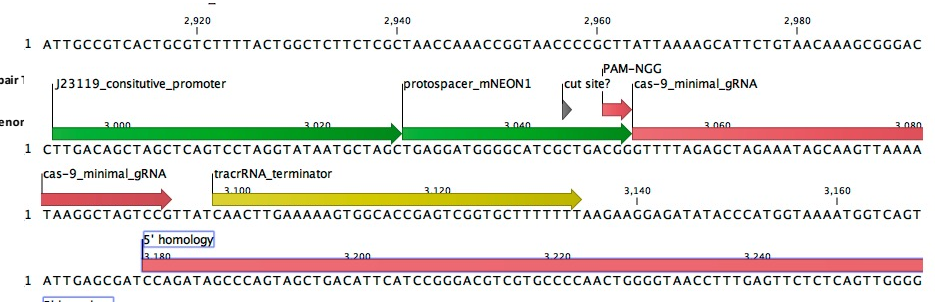
CRISPR Installation

Task 2: Develop Cas9 mediated editing in *C. necator*

- ***Vectors and Cas9/GFP expressing strains were delayed due to lack of available researcher***
- ***Recently recruited a student intern at NREL to resume task***
- ***Cas9-based editing was demonstrated for C. necator***







Gene Translational Control Elements

Task 3: Develop Ribosome Binding Sequence library for *C. necator*

- An *in silico* RBS calculator* developed by AGILE was used to create a library of synthetic RBSs with broad strength range

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                    10        20        30        40        50        60        70        80        90
                    ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|-
Consensus           GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGUCUCAAAAGGAGGUAUCUUUAUGGCGAGUAGCGAAGACGUUAUCAAAGAGUUCAU
cal_rbs_RFP1        GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGgCUCAAAAGGAGuUAgCcccAUGGCGAGUAGCGAAGACGUUAUCAAAGAGUUCAU
cal_rbs_RFP2        GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGUCUCAgggaGAGGUccgaaaAUGGCGAGUAGCGAAGACGUUAUCAAAGAGUUCAU
cal_rbs_RFP3        GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGUuUCAguAGGAGGcAaCcaUAUGGCGAGUAGCGAAGACGUUAUCAAAGAGUUCAU
cal_rbs_RFP4        GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGaaaagAggaGgGagAagcUUAUGGCGAGUAGCGAAGACGUUAUCAAAGAGUUCAU
cal_rbs_RFP5        GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGaCUuugAAGGgGGcAgaUccAUGGCGAGUAGCGAAGACGUUAUCAAAGAGUUCAU
cal_rbs_RFP6        GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGgaUCAAAAGGcuGggUuUUUAUGGCGAGUAGCGAAGACGUUAUCAAAGAGUUCAU
cal_rbs_RFP7        GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGUucCAAAAGGAGGcccCggcAUGGCGAGUAGCGAAGACGUUAUCAAAGAGUUCAU
cal_rbs_RFP8        GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGaagaAAAAGGucGguUuUUUAUGGCGAGUAGCGAAGACGUUAUCAAAGAGUUCAU
cal_rbs_RFP9        GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGUaagcgcgGGAGGUAUuaUaAUGGCGAGUAGCGAAGACGUUAUCAAAGAGUUCAU
cal_rbs_RFP10       GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGcCUCAAAAGGAGGUAgCUUaAUGGCGAGUAGCGAAGACGUUAUCAAAGAGUUCAU
cal_rbs_RFP11       GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGcCcCcAAAGGAGGUAgCUaUAUGGCGAGUAGCGAAGACGUUAUCAAAGAGUUCAU
cal_rbs_RFP12       GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGUCUCAcgAGGAGGUuUaUUaAUGGCGAGUAGCGAAGACGUUAUCAAAGAGUUCAU
cal_rbs_RFP13       GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGUCcggAAAGGAGGUAcgcaaAUGGCGAGUAGCGAAGACGUUAUCAAAGAGUUCAU
cal_rbs_RFP14       GGAGACCACAACGGUUUCCCUCUAGAAAUAAUUUUGagagcuAAGGAGGUuUaUUUAUGGCGAGUAGCGAAGACGUUAUCAAAGAGUUCAU
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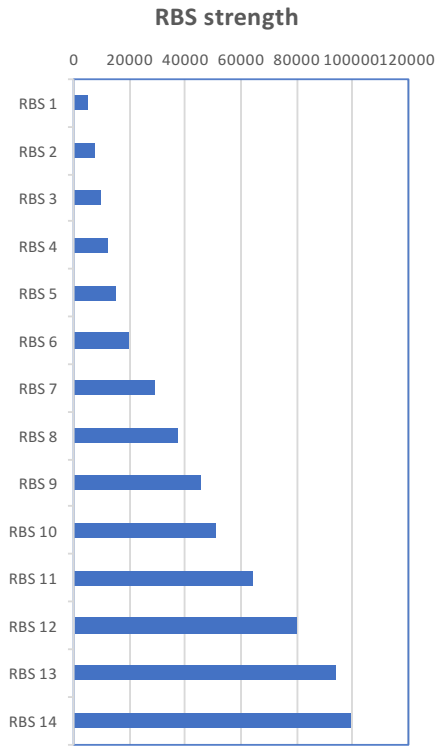


Cal rbs rfp region input seq

De novo seq

RFP input seq

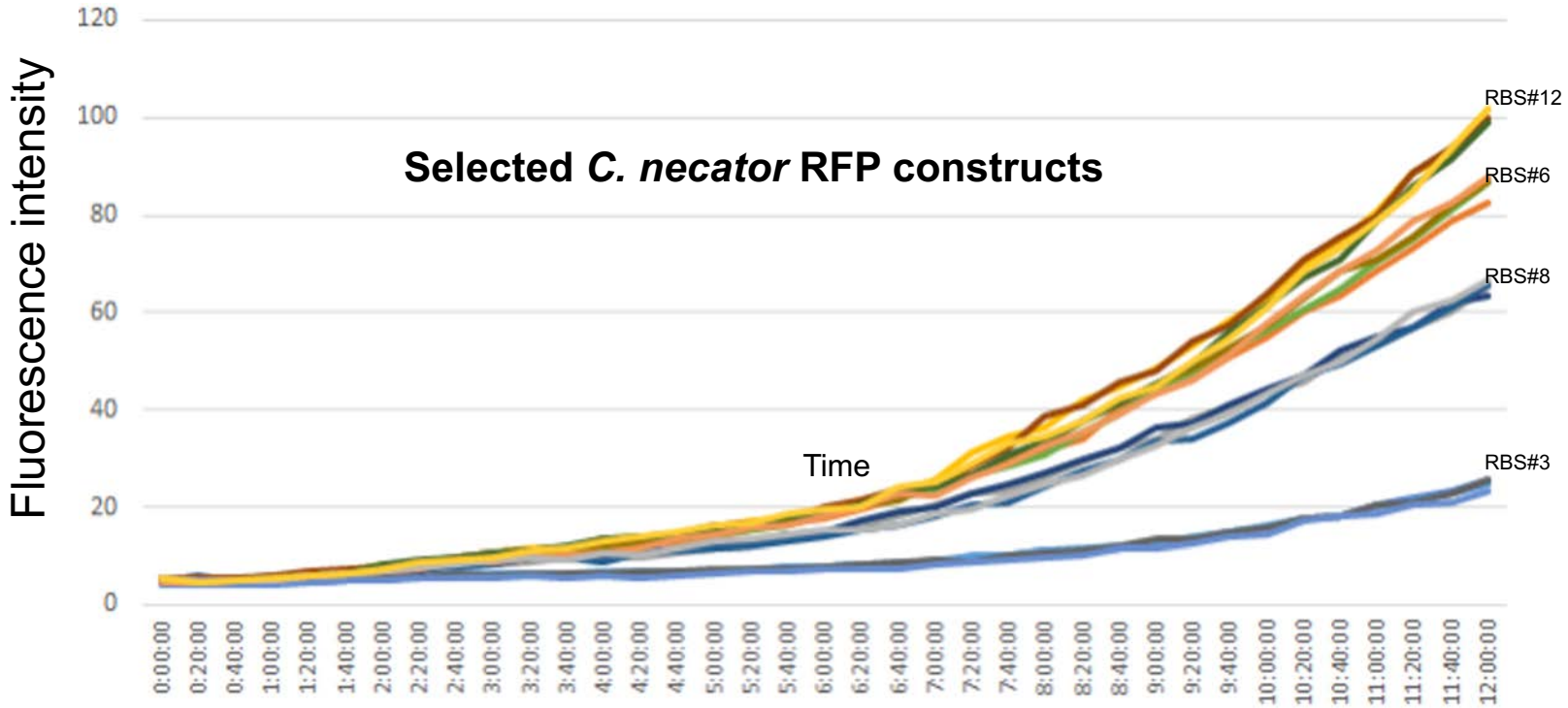
Bi, C., Su, P., Müller, J. *et al.* (2013) Development of a broad-host synthetic biology toolbox for *Ralstonia eutropha* and its application to engineering hydrocarbon biofuel production. *Microb Cell Fact* **12,** 107

Gene Translational Control Elements

Kiverdi

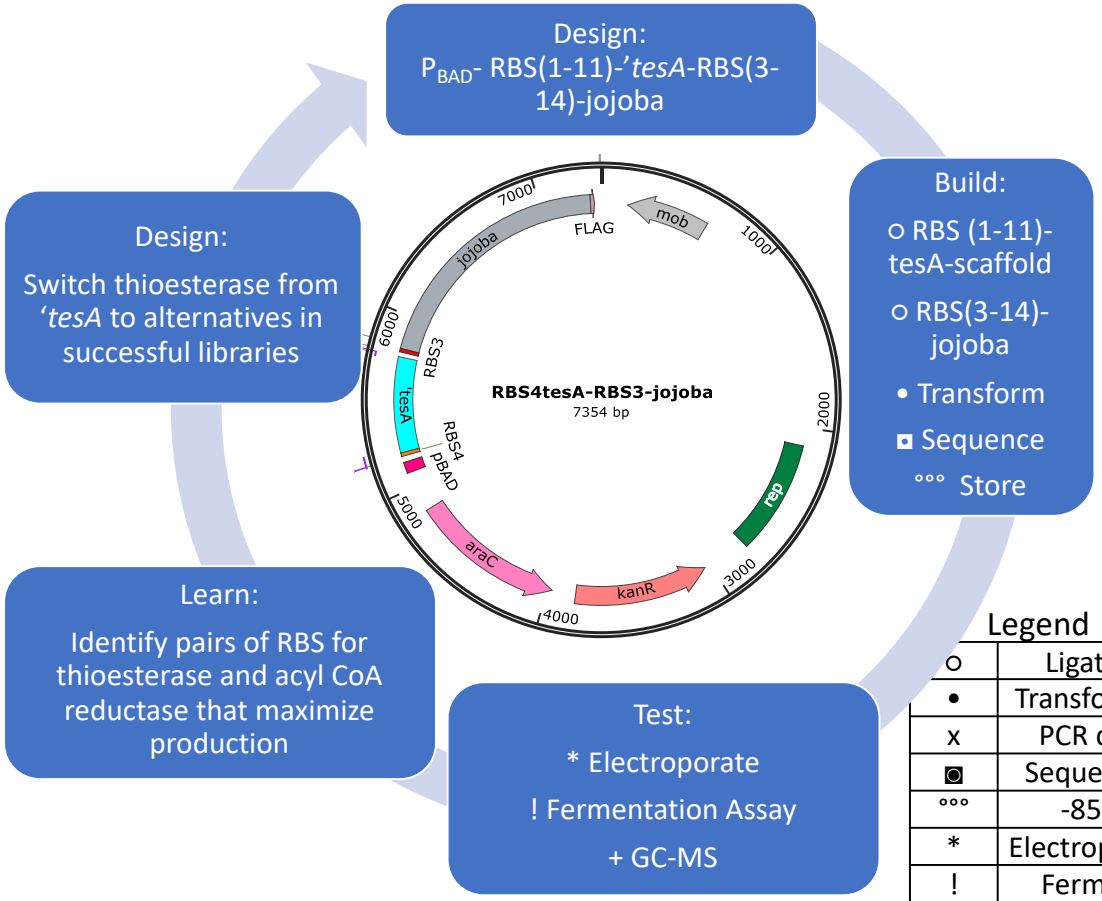
Task 3: Develop RBS library for *C. necator*

- Plasmids for 14 distinct synthetic RBSs were constructed by AGILE DiVA team
- RFP fluorescence was roughly correlated with predicted RBS expression strength



Design-Build-Test for Dodecanol

Task 4: 1-dodecanol production



Legend

○	Ligation
•	Transformed
x	PCR conf
◘	Sequenced
°°°	-85°C
*	Electroporate
!	Ferment
+	GC-MS

jojoba RBS	tesA RBS 1	2	3	4	5	6	7	8	9	10	11
3	○	◘	○	◘	○		◘	○	○	◘	
4	○	◘	○	◘	○	○	◘	○	○	◘	
5	○	◘	○	◘	○	○	◘	○	○	◘	
6	○	◘	○	◘	○	○	◘	○	○	◘	
7		◘	○	◘	○	○	◘	○	○	◘	
8		◘	○	◘	○	○	◘	○	○	◘	
9		◘	○	◘	○	○	◘	○	○	◘	
10		◘	○	◘	○	○	◘	○	○	◘	
11		◘	○	○	○	◘	○	○	◘	◘	
12	○	◘	○	○	○	◘	○	○	◘	◘	
13	○	◘	○	○		◘	○	○	○	◘	
14	○	◘	○	○		◘	○	○	◘	○	
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Table. Current status of strains construction and analysis

1. Project Milestones

Task 1: **Method(s) for 10-fold improved transformation efficiency in *C. necator* (ORNL) (Complete)**

Subtask	Due	% fin	Issues if any
Improve transformation efficiency of WT *C. necator* H16 at least 10-fold	9/30/19	100%	

Task 2: **Developing CRISPR/Cas9 editing in *C. necator* (NREL)**

Subtask	Due	% fin	Issues if any
Identify at least 1 functional CRISPR/Cas system for gene editing	9/30/20	100%	
Demonstrate simultaneous editing of at least two genes	3/31/21	50%	
CRISPR-based editing with pooled library >4 mutations	9/30/21	15%	

2. Project Milestones

Task 3: Develop RBS library for *C. necator* (LBNL/Kiverdi) (Complete)

Subtask	Due	% fin	Issues if any
Identify at least 10 RBSs to be tested	6/30/19	100%	
RFP readouts for 10 RBSs in *C. necator*	3/31/21	100%	

Task 4: 1-Dodecanol production (LBNL/Kiverdi)

Subtask	Due	% fin	Issues if any
Plasmids and background *C. necator* strain developed for dodecanol production	9/30/20	100%	
Transform *C. necator* with RBS variant plasmids	12/31/20	100%	
Measure dodecanol production in strain variants	3/31/20	45%	

3. Project Milestones

Task 5: **Autotrophic Production of 1-Dodecanol (Kiverdi)**

Subtask	Due	% fin	Issues if any
Optimize *C. necator* fermentation with H_2/CO_2	6/30/21	0%	

Task 6: **Chromosomal integration of dodecanol pathway (LBNL/Kiverdi/ORNL)**

Subtask	Due	% fin	Issues if any
Integration of 1-dodecanol pathway into *C. necator* chromosome	3/31/21	10%	
Demonstration of 1-dodecanol production from chromosomal pathway	6/30/21	0%	
Measure dodecanol production in strain variants	9/30/21	0%	

Next Steps

1	**Task 2: Develop Cas9 mediated editing in C necator**
	Demonstrate multi-gene disruptions with Cas9
	Target *C. necator* thioesterases to improve 1-dodecanol production
2	**Task 4: 1-dodecanol production**
	Test 1-dodecanol production under heterotrophic conditions
	Completion of RBS set for '*tesA* and jojoba *acr*
	Replacement of *'tesA* with other thioesterases; overexpress native *fadD* to improve titers
3	**Task 5: Autotrophic Production of 1-Dodecanol**
	Most promising strains from heterotrophic growth will be tested under H_2/CO_2
4	**Task 6: Chromosomal integration of dodecanol pathway**
	Choice of thioesterase and acyl CoA reductase genes from plasmid-based expression
	Integration of genes using phage integrase method

Project Issues

Issue	Approach
• Pandemic restrictions have prevented Kiverdi from performing on-site work at LBNL	LBNL increased scope on project to transfer additional engineering tasks
• General delays because of pandemic restrictions	NCE until 9/30/21 was implemented and milestones were rescheduled

Summary

Key Takeaways

- Superior electroporation protocol for DNA transformation attaining 10^7 cfu/μg DNA
- Developed 4 highly efficient site-specific recombinases for *C. necator*
- Developed chromosomal promoter library with 400-fold expression range
- 600-fold expression range from chromosomal, nitrogen-limitation inducible T7 system
- Developed broad library of RBS expression control elements
- DBTL cycle initiated for 1-dodecanol production, will include heterotrophic and autotrophic growth

Publication

- George L. Peabody, Joshua R. Elmore, Jessica Martinez-Baird, Gara N. Dexter, and Adam M. Guss “Enhanced synthetic biology toolkit for *Cupriavidus necator* H16” *Metabolic Engineering*, in revision

Commercialization potential

- Kiverdi will test 1-dodecanol production strains in bioreactors and assess commercial potential for dodecanol production from CO_2

Quad Chart Overview

Timeline

- Start 1/1/2019
- End 9/30/2021 (Extended)

	Y1	Y2
DOE Funding	$450,000	$450,000
Cost Share	$192,800	$192,800
Total	$642,800	$642,800

Project Partners

- Kiverdi, LBNL, ORNL, NREL

Barriers addressed

Advanced Bioprocess Development; engineering robust organisms and fermentation regimes for sustainable production of bioproducts

Project Goal

Develop tools for metabolic engineering of model chemolithoautotrophy and demonstrate these tools through autotrophic production of 1-dodecanol

End of Project Milestone

As a concrete demonstration of this proposed platform's potential capabilities, we will engineer *C. necator* to convert H_2 and CO_2 into the C_{12} fatty alcohol, 1-dodecanol (lauryl alcohol), commonly used in the production of surfactant and detergent products and currently sourced from palm kernel oil, the cultivation of which is associated with widespread rainforest destruction.

Agile Biofoundry CRADA FP00006779

Additional Slides

Publications

George L. Peabody, Joshua R. Elmore, Jessica Martinez-Baird, Gara N. Dexter, and Adam M. Guss “Enhanced synthetic biology toolkit for *Cupriavidus necator* H16” *Metabolic Engineering*, in revision