Agile BioFoundry

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

BIOENERGY TECHNOLOGIES OFFICE

Agile Genetics for Biomanufacturing

Ellen L. Neidle
University of Georgia

DOE Agile BioFoundry

BETO Peer Review 2021
Conversion Technologies
March 10, 2021
1:45 pm (Eastern time)

Project overview

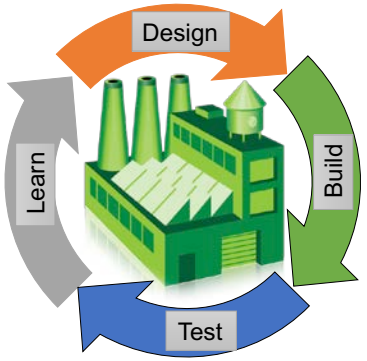
Develop new adaptive lab evolution methods to increase the microbial synthesis of valuable end products

- **Goal**: harness evolution for metabolic engineering; use synthetic biology to select strains with maximal production (or degradation) of target molecules
- **Bacterial Host**: *Acinetobacter baylyi* ADP1 allows powerful DNA manipulation in ways not possible in other organisms
- **Target Molecule, TPA**: commodity chemical in high demand; PET plastic waste accumulates in the environment making biodegradation important also

Evolution:
The Ultimate Natural DBTL Cycle

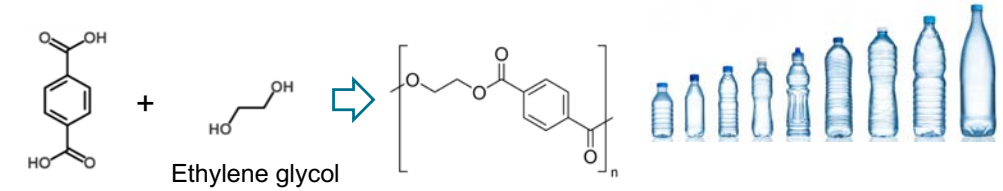
Biological Process

Design: Genetic Change
Build: Phenotypic Expression
Test: Fitness Competition
Learn: Selection



Terephthalic acid (TPA): commercial importance

- bioproduction from renewable biomass feedstock
- biodegradation to reduce plastic waste



Terephthalic acid (TPA)

Ethylene glycol

Polyethylene terephthalate (PET)

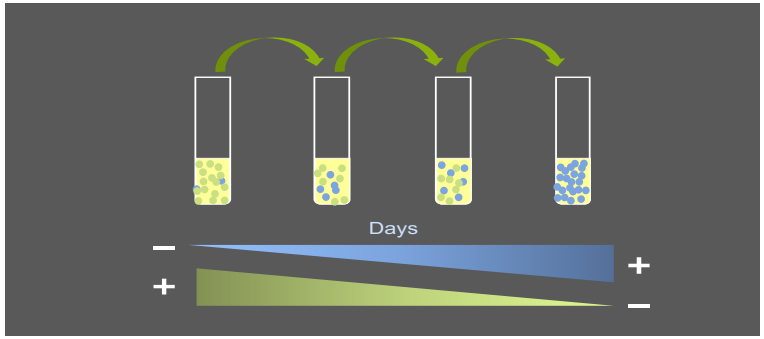
>30 M tons global capacity (2017)

$1000-$3000 per ton

Project overview

Develop new adaptive lab evolution methods to increase the microbial synthesis of valuable end products

- **Current method of adaptive lab evolution?** Serial transfer of evolving population selects faster growing cells



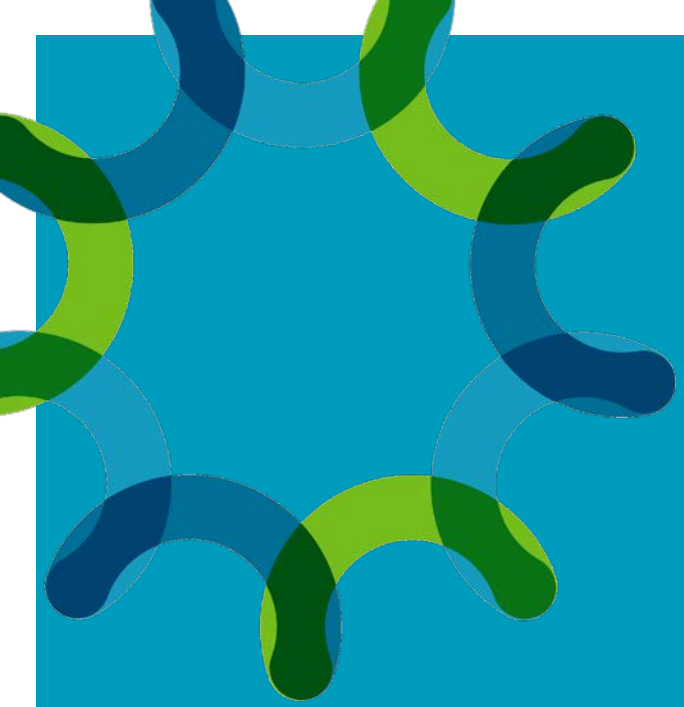
Serial transfer

the % of fast-growing cells (blue) increases while that of slow-growing cells (green) decreases

- **Limits of current methods?** Growth is at best an indirect measure of improved metabolic performance

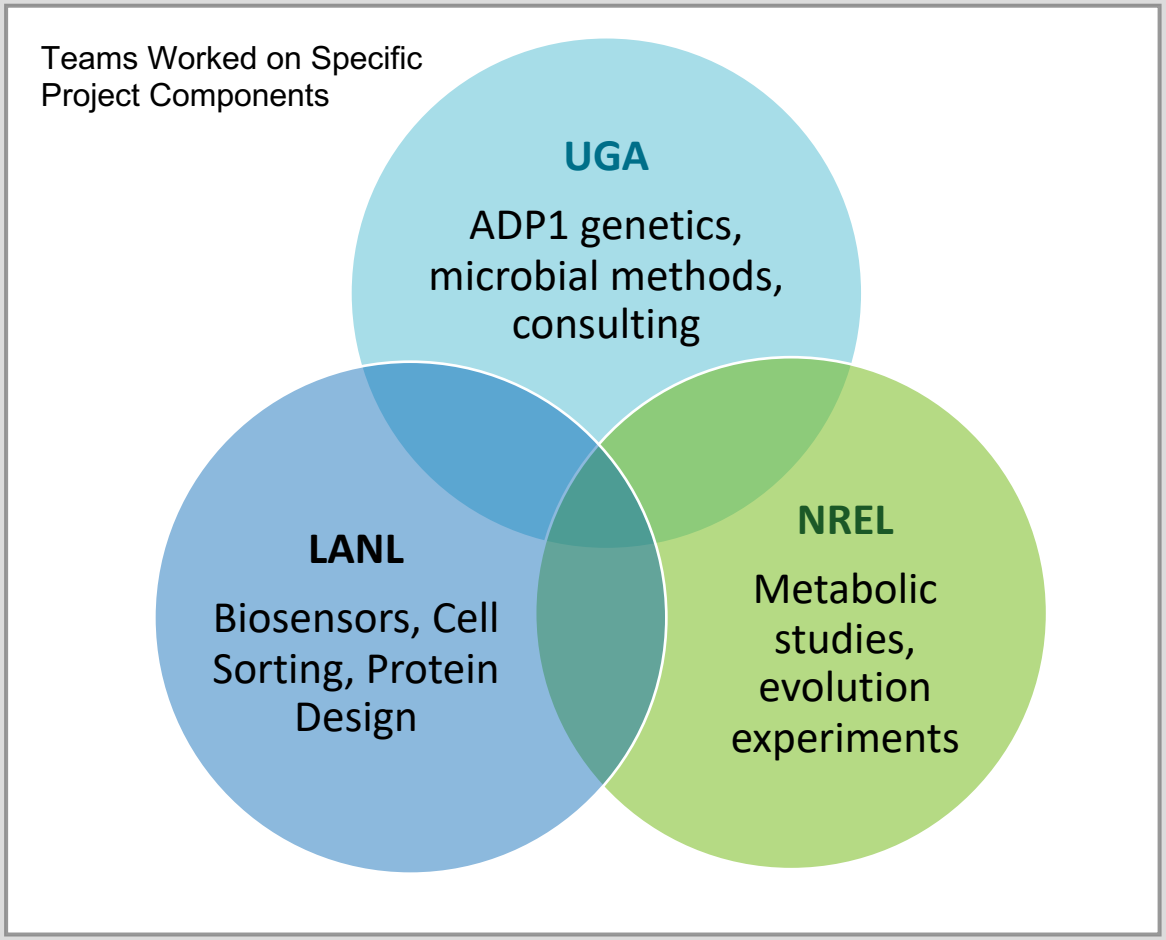
- **Importance of improving techniques?** Biomanufacturing requires maximal product synthesis. Combining adaptive evolution with methods to select strains with improved production represents a significant paradigm shift for the bioeconomy.

- **Risks?** Biological method development is typically a slow process characterized by unanticipated obstacles. This project involves novel and untried approaches, and challenging metabolic reactions



1 - Management

Project Structure



UGA

Ellen Neidle
Alyssa Baugh
Stacy Bedore

NREL

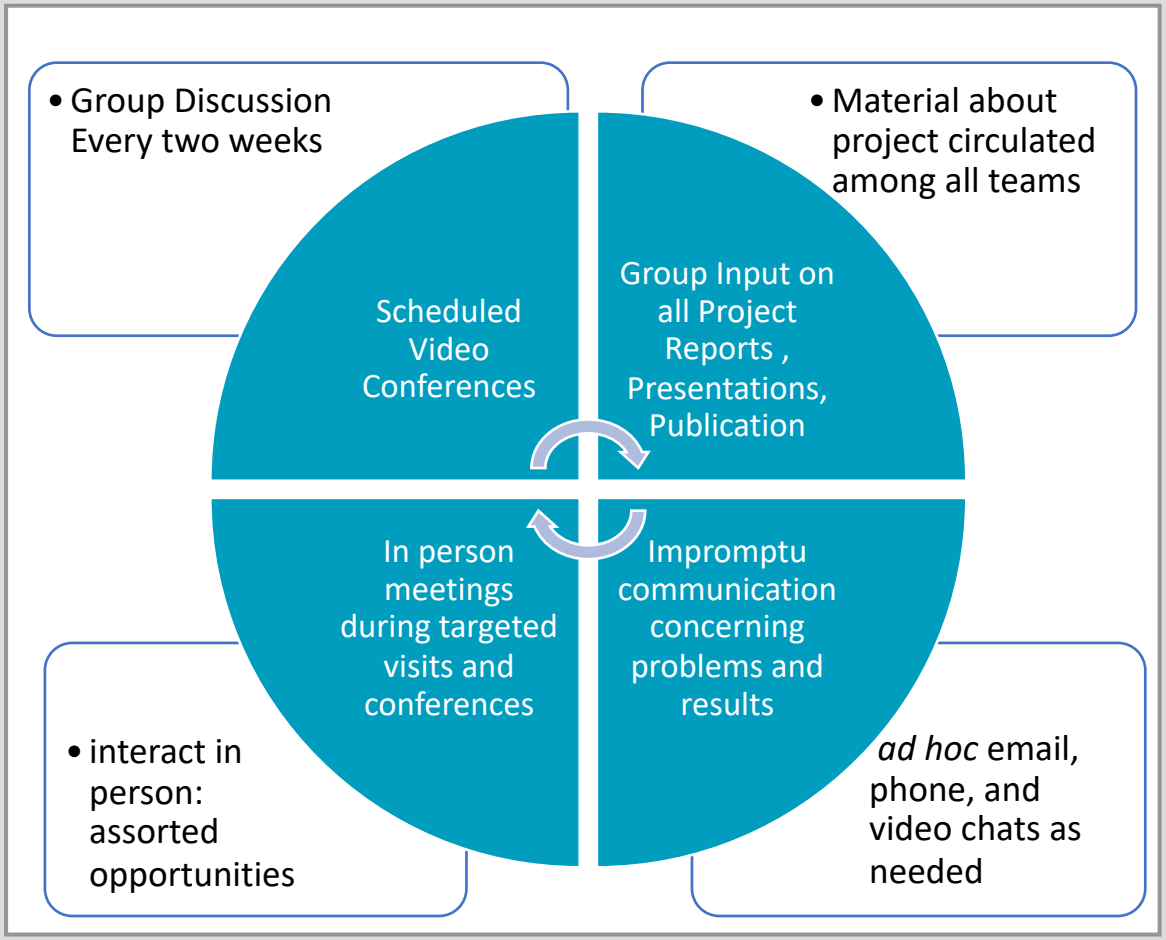
Christopher Johnson
Gregg Beckham
Isabel Pardo Mendoza
Felicia Bratti, Molly Gaddis

LANL

Ramesh Jha
Taraka Dale
Ryan Bermel

Workforce Development: Mix of postbac students, graduate students and postdocs

Communication and Collaboration



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Risk Mitigation

Problems discussed and assessed in scheduled group meetings. Three main mitigation strategies:

Work on Project Components in Parallel	• Experimental plan broken into parts that can be completed independently
Alternative Strategies for Key Parts	• Discussed and planned backup strategies: Some of these were implemented
Expertise to Pivot and Focus on Unexpected Results	• Prepared to move in new directions quickly, as this is often needed for evolutionary research

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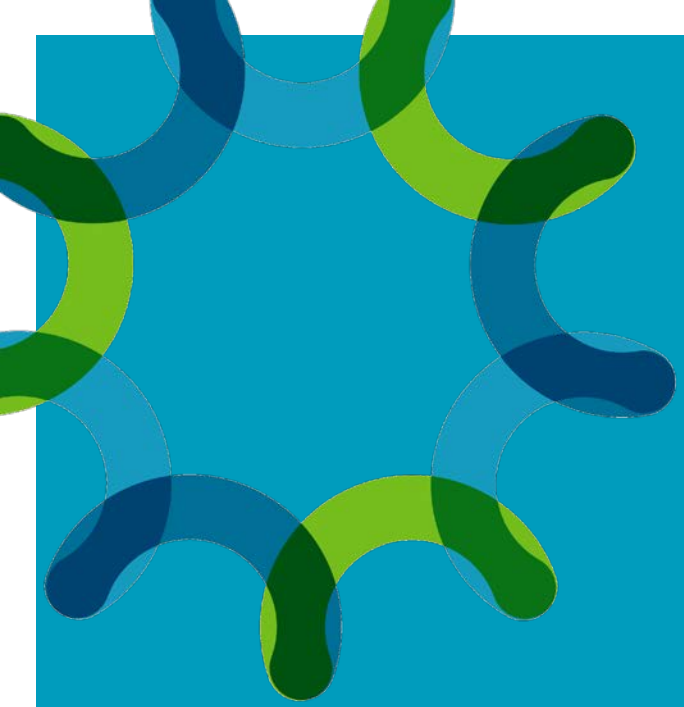


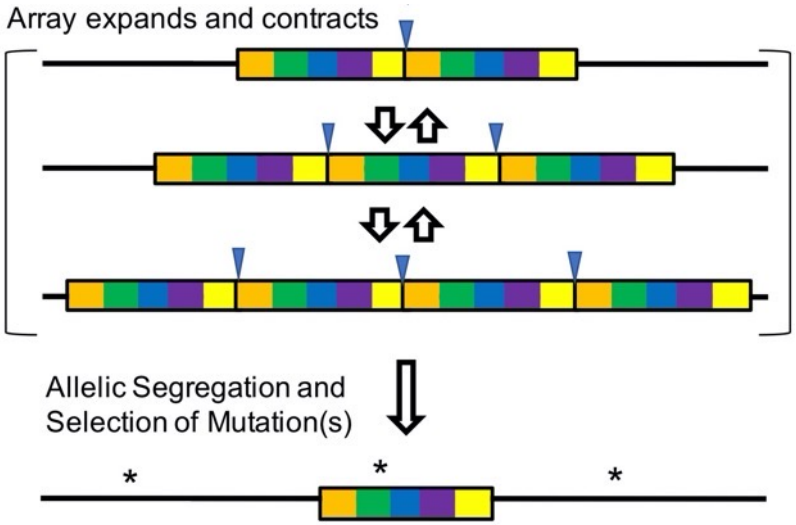
2 - Approach

Approach: Build on a Novel Evolutionary Method Developed by Team Members from UGA and NREL

Evolution by Amplification and Synthetic Biology (EASy)

Multiple gene copies can confer new phenotypes

Beneficial mutations arise that obviate the need for multiple copies



Adaptive lab evolution: maintain selection for new phenotypes during serial transfers
Method accelerates selection of new traits by increasing chromosomal copy number

PNAS

Accelerating pathway evolution by increasing the gene dosage of chromosomal segments

Melissa Tumen-Velasquez[a,1], Christopher W. Johnson[b,1], Alaa Ahmed[a], Graham Dominick[b], Emily M. Fulk[b], Payal Khanna[b], Sarah A. Lee[c], Alicia L. Schmidt[a], Jeffrey G. Linger[b], Mark A. Eiteman[a,c], Gregg T. Beckham[b,2], and Ellen L. Neidle[a,2]

[a]Department of Microbiology, University of Georgia, Athens, GA 30602; [b]National Bioenergy Center, National Renewable Energy Laboratory, Golden, CO 80401; and [c]School of Chemical, Materials and Biomedical Engineering, University of Georgia, Athens, GA 30602

https://doi.org/10.1073/pnas.1803745115

Approach: Alter EASy method to select for increased product instead of rapid growth

EASy Method Issues

GOOD

- Changes not predictable by rational design
- Can select under diverse conditions (including temperature, pH, etc.)
- Mutations in unexpected genes

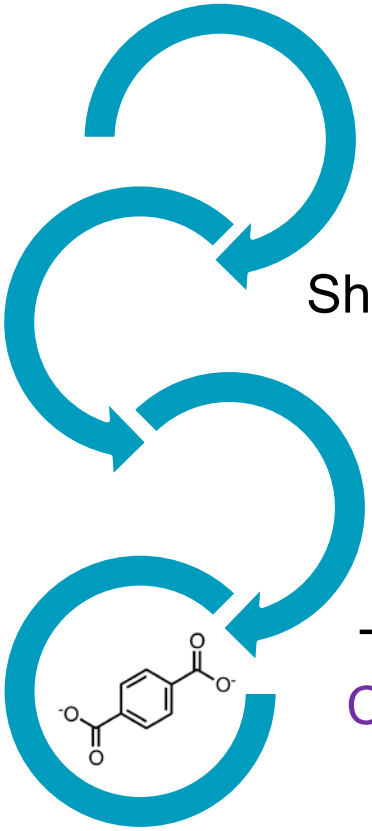
BAD

- Mutations in unexpected genes
- Growth is an indirect measure of end-product synthesis (and may negatively correlate with desired outcome)

Address this problem using biosensors for this project

Approach: Design pathway to be engineered in ADP1

Apply EASy-based Methods to TPA metabolism

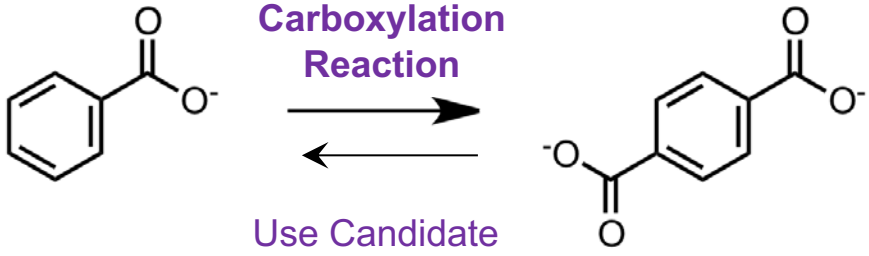


Biomass to Sugars

Shikimate Pathway to Synthesize Aromatic Compounds (Benzoate)

TPA production Carboxylation of Benzoate

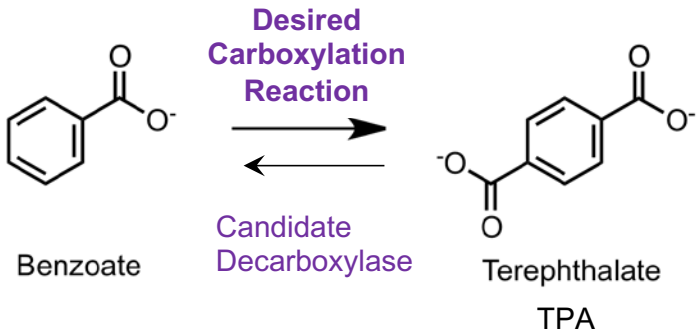
TPA



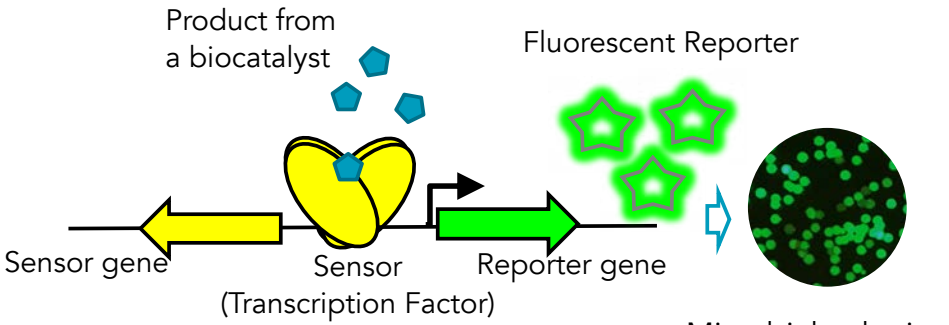
Most enzymes favor decarboxylation

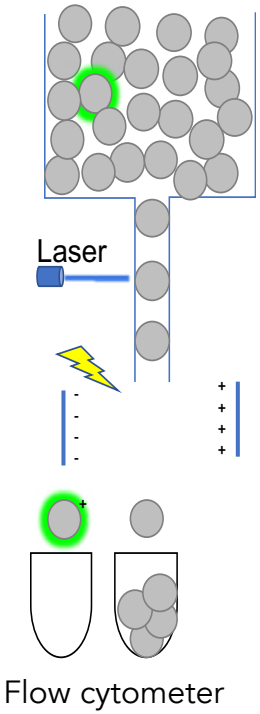
Candidate Decarboxylase: No known enzyme with this activity, so protein engineering and evolution will be used to alter substrate specificity of a bacterial enzyme with similar activity

Approach: Develop new biosensors to detect benzoate and TPA



Most such enzymes favor decarboxylation





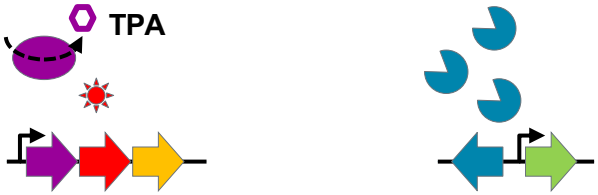
Detect strains that produce TPA using a new biosensor

Approach: Combine EASy and Biosensors

Proof-of-concept: Reversible decarboxylase for the biosynthesis of terephthalate

Evolve Enzyme to Produce TPA

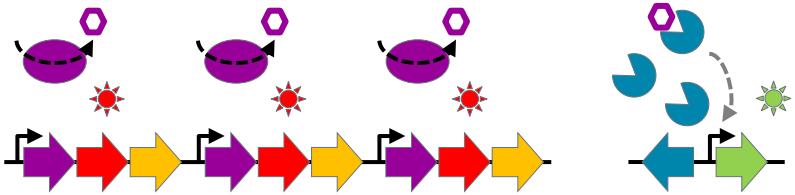
Red Fluorescent Protein (RFP)
Indicates gene dosage



Biosensor: Detects TPA responds to increase Green Fluorescent Protein (GFP)

Low copy number → **Low RFP**

Low activity → **Low GFP**



High copy number → **High RFP**

Low activity → **Low GFP**

EASy amplification, multiple weak enzymes increase TPA production

Low copy number → **Low RFP**

High activity → **High GFP**

After evolution improved enzyme produces TPA

Fluorescence and ratio of RFP/GFP signal for fluorescence-activated cell sorting (FACS) by flow cytometry

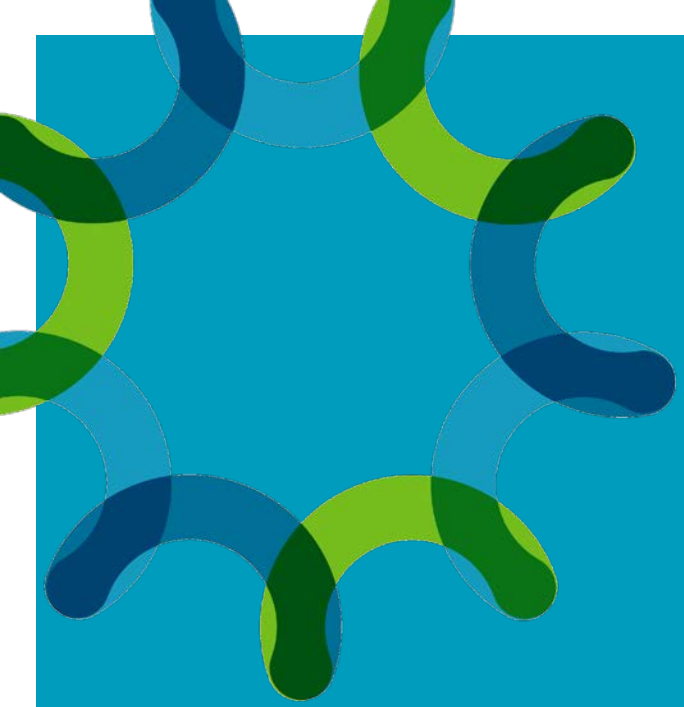
Approach: challenges and risk mitigation

Challenges	Mitigation and alternative strategies
• No enzyme known to carboxylate benzoate to TPA; difficult to evolve an enzyme to catalyze this reaction	• Enzyme may function more efficiently in the direction of decarboxylation, can improve this function- learn more about enzyme; **TPA degradation is also important**
• Don't know about TPA transport in and out of ADP1	• Conduct experiments in parallel to improve understanding of transport
• Difficult to develop all cell sorting and gene amplification methods simultaneously	• Work on individual steps: progress on different steps could result in major advances

Approach: metrics for progress*

Milestones	Rationale
(1) Demonstrate detection of 0.5 mM TPA by a biosensor.	Biosensor development is a critical step of method
(2) Demonstrate multiple copies of chromosomal target region with qPCR; amplified region 3X or greater	Shows EASy method working to produce sufficient enzyme for accelerated evolutionary selection.
(3) Demonstrate time-dependent changes in GFP/RFP ratios	Basis of novel methodology; Shows GFP/RFP ratios can be used to sort cells according to product
(4) Document a TPA titer of 100 mg/L	Production goal

***Go/No-Go not discussed since project has ended and No-Go points were never reached**



3 - Impact

Impact

Biosensor Development

- Biosensors provide sensitive detection of metabolites; instrumental for improving metabolic engineering
- TPA sensor: importance of TPA as a commodity chemical and as a degradation product of plastic wastes suggests broad applications for this new resource
- Benzoate sensor: provides critical tool for studies of decarboxylase/carboxylase reactions for TPA degradation and synthesis; Class of enzyme underutilized in biomanufacturing and has exciting biotechnology applications

Development of ADP1 as a robust microbial chassis for wide range of protein engineering schemes

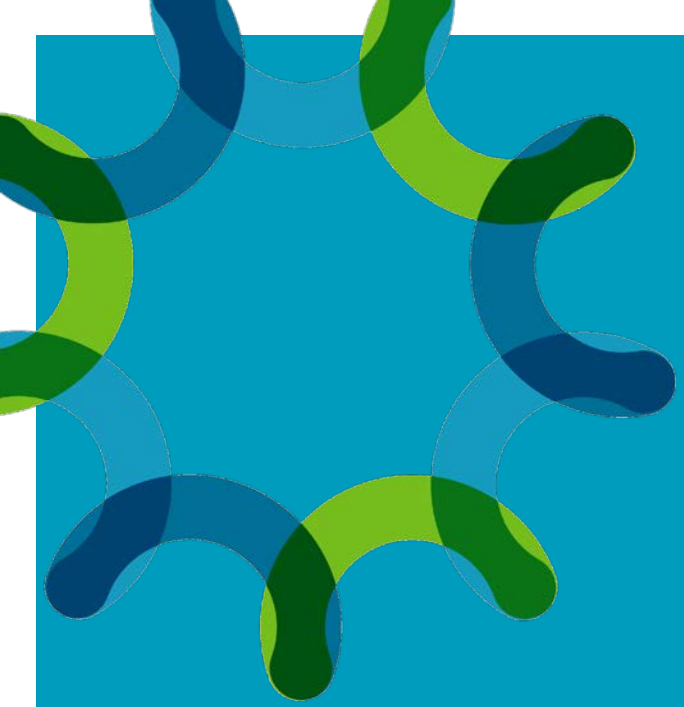
- EASy methodology and genetic system of ADP1 provide unique opportunities for bioengineering

Commercialization potential

- TPA production and degradation are industrially important

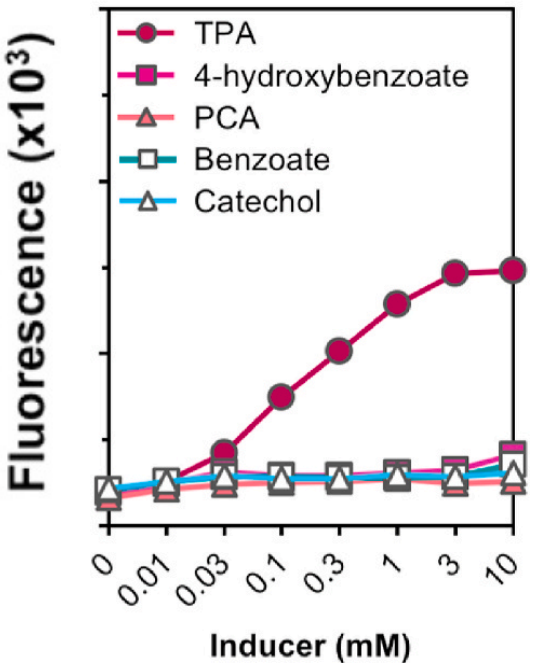
Results Dissemination

- Publication in Metabolic Engineering (2020, Pardo & Jha, et al., Metabolic Engineering, https://doi.org/10.1016/j.ymben.2020.09.009
- Two provisional patents submitted



4 - Progress and Outcomes

New tools and reagents for TPA synthesis and degradation using *Acinetobacter baylyi* ADP1 as a microbial chassis



The TPA biosensor enabled dose-dependent detection of TPA but not related molecules 4-HB, PCA, benzoate, and catechol.

TPA Biosensor Developed

Results published

Metabolic Engineering 62 2020) 260–274

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/meteng

Original Research Article

Gene amplification, laboratory evolution, and biosensor screening reveal MucK as a terephthalic acid transporter in *Acinetobacter baylyi* ADP1

Pardo & Jha, et al., *Metabolic Engineering*, 2020

https://doi.org/10.1016/j.ymben.2020.09.009

- A novel TPA biosensor (pTPA3) was developed that detects TPA specifically and in a dose-dependent fashion (milestone 1)

New tools and reagents for TPA synthesis and degradation using *Acinetobacter baylyi* ADP1 as a microbial chassis

Conducted experiments to improve understanding of TPA transport

Results published

Pardo & Jha, et al., *Metabolic Engineering*, 2020

https://doi.org/10.1016/j.ymben.2020.09.009

- Using EASy, ADP1-derived strains expressing foreign genes were isolated that rapidly consume TPA as a sole carbon source. This TPA degradation was better than that of other bacteria that naturally grow on TPA.
- Our biosensor helped reveal that that a transporter in ADP1 known to transport muconate (MucK) can also transport TPA. Mutations in evolved strains revealed variant proteins that improve TPA uptake in ADP1.
- Two provisional patents related to this work have been submitted

<u>Degradation of TPA has broader importance for addressing problems of plastic wastes</u>.

Progress made on Combining EASy with Biosensor-Based Detection of Products

Milestone 2 was met

(2) Demonstrate multiple copies of chromosomal target region with qPCR; amplified region 3X or greater

Milestone 3 was partially met

((3) Demonstrate time-dependent changes in GFP/RFP ratios

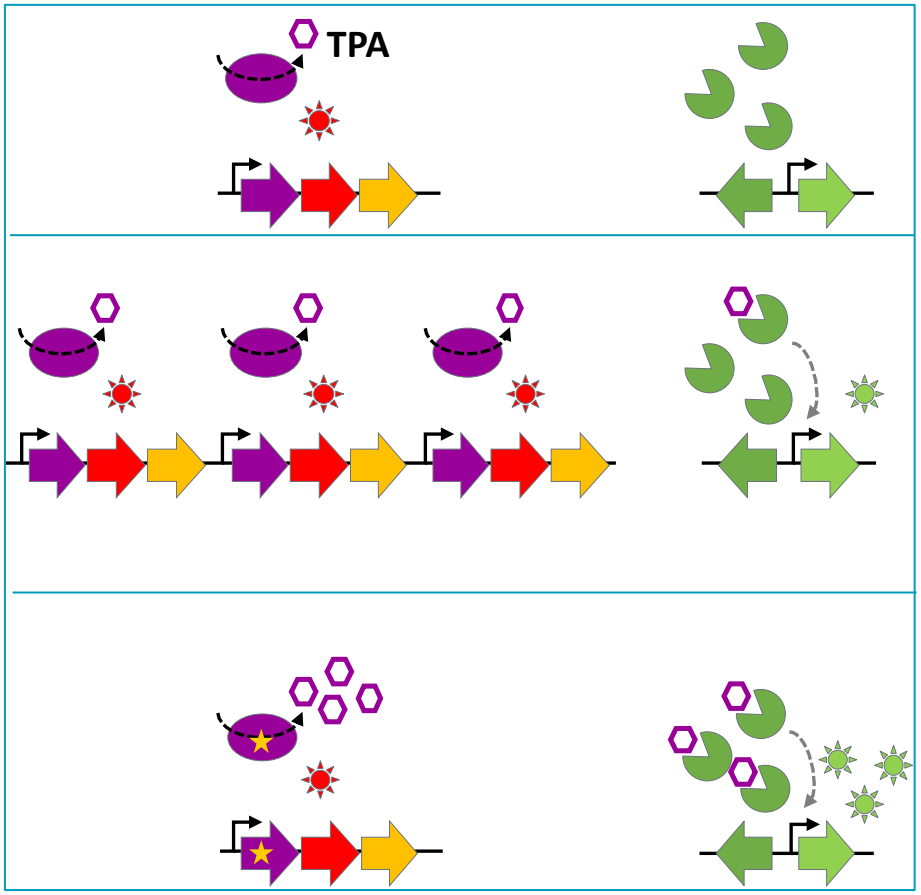
Obstacle was low level carboxylation to form TPA. Carboxylation-Decarboxylation catalysis requires further study

Milestone 4 was not met

(4) Document a TPA titer of 100 mg/L

Obstacle was low level carboxylation to form TPA. Carboxylation-Decarboxylation catalysis requires further study

Novel approach remains promising.



Ratiometric sorting worked, but further development of this method is needed.

Key challenge: enzymatic carboxylation of benzoate to produce TPA

Summary

- **Significant Results**:
  - Biosensors developed for TPA and benzoate
  - *A. baylyi* ADP1 further developed as a synthetic biology chassis
  - TPA-degrading ADP1-derived strains were generated
  - New information about TPA transport proteins emerged
  - Methods for experimental evolution were advanced

Quad Chart Overview

Timeline

- Start: October 1, 2018
- End: Dec 30, 2020

	FY19	FY20	Total Active
LANL	$140,000	$140,000	$280,000
NREL	$110,000	$110,000	$220,000
Total DOE	$250,000	$250,000	$500,000
UGA Cost Share	$107,100	$107,100	$214,200
TOTAL CRADA	**$357,100**	**$357,100**	**$714,200**

Project Goal

Develop new adaptive lab evolution methods to increase the microbial synthesis of valuable end products, with TPA as target molecule

End of Project Milestones

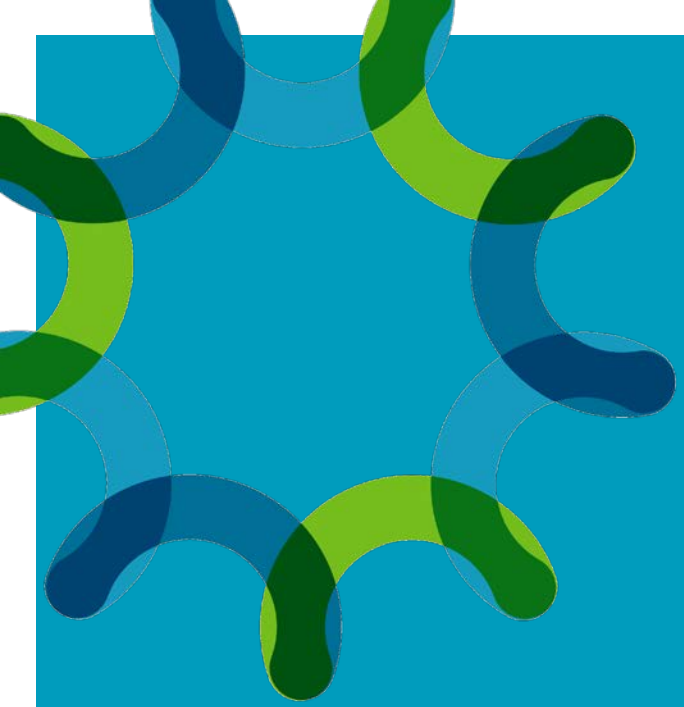
- Demonstrated that multiple copies of chromosomal target region can be assessed using fluorescence: qPCR and RFP measurements correlate
- time-dependent changes in GFP/RFP ratios indicate that methodology is working
- TPA titer not measured: problem with attaining sufficient carboxylation of benzoate

Project Partners

- LANL (40%)
- NREL (30%)
- UGA

Funding Mechanism

2017 ABF DFO



Additional Slides

Responses to Previous Reviewers' Comments

2019 Project Review: in 2019, the project was just starting. A Poster was presented showing progress from first few months of research. There were no reviewer comments to address

Publications, Patents, Presentations, Awards, and Commercialization

Publication

Gene amplification, laboratory evolution, and biosensor screening reveal MucK as a terephthalic acid transporter in *Acinetobacter baylyi* ADP1, *Metabolic Engineering,* October 2020, https://doi.org/10.1016/j.ymben.2020.09.009

Presentations

Neidle E, "Agile Evolution Made Easy" at the "*Accelerating the Bioeconomy*" ABF/NSF sponsored workshop, February 2020

Jha RK, "Biosensor-mediated Biocatalyst Engineering"; Invited talk, U. Portsmouth, UK, November 2020

Commercialization

Provisional patent application submitted at LANL, February 2020
LANL S133844 - Terephthalate Biosensor & Applications Thereof

Provisional patent application submitted at NREL, September 2020
NREL PROV 20-53 - Mutant transporters for bacterial uptake of terephthalic acid

Award

Dr. Ramesh Jha (LANL, lead investigator) received a R&D 100 award for "Smart Microbial Cell Technology" for the ultra-high-throughput screening platform to engineer custom biocatalyst (technology incorporated in this project)
https://www.lanl.gov/discover/news-release-archive/2020/October/1005-rd100-awards.php