DOE Bioenergy Technologies Office (BETO)
2021 Project Peer Review

Engineering multi-gene CRISPRa/i programs to accelerate DBTL cycles in ABF Hosts

Thursday, March 11, 2021
Technical Session Review Area: Agile BioFoundry Consortium

James M. Carothers, Ph.D.
University of Washington

James Carothers (PI)
Jesse Zalatan
Herb Sauro

Rob Egbert
Alex Beliaev
Jon Magnusson (ABF)

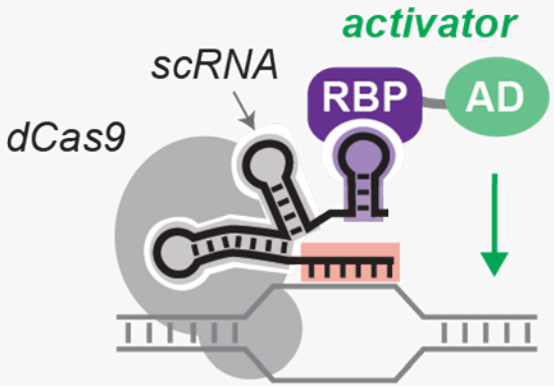
Hector Garcia Martin (ABF)

Project Overview

Engineering multi-gene CRISPRa/i programs to accelerate DBTL cycles in ABF Hosts

1. CRISPRa tools

CRISPRa (Activation)

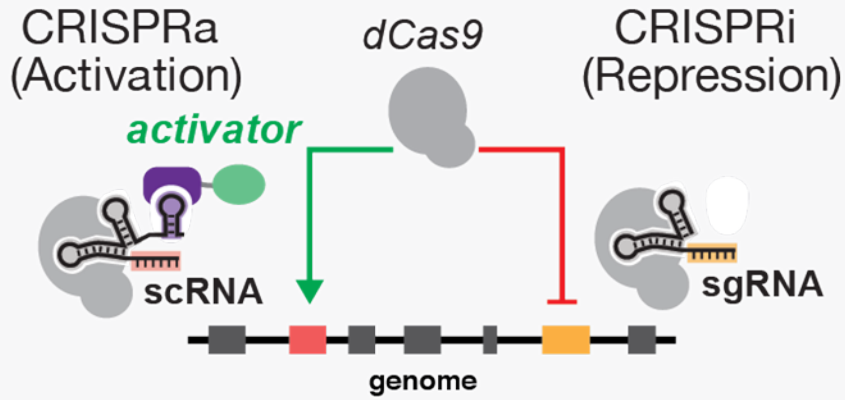


modified guide RNA (scRNA) recruits activation domain

ABF host	CRISPRa status
P. putida	Demonstrated
C. glutamicum	Proposed
B. subtilis	Demonstrated
S. elongatus	Proposed
Z. mobilis	Proposed

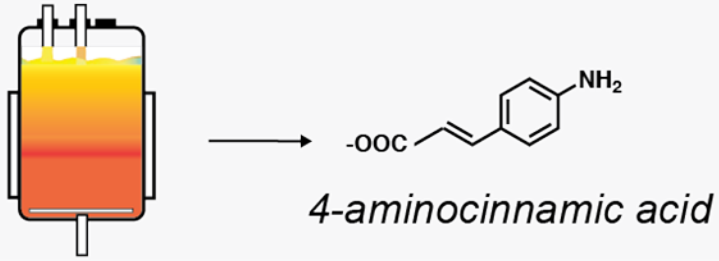
2. Transcriptional programs

Multi-Gene Programs



scRNA expression directs CRISPRa
sgRNA expression directs CRISPRi

3. Industrial aromatic production



4-aminocinnamic acid

Summary

Develop CRISPR-Cas expression tools:
- Create new abilities to activate bacterial gene expression in ABF hosts
- Create platforms for design-driven multi-gene expression tuning

Combine with ABF capabilities:
- Multiomics technologies (PNNL)
- Machine learning (LBNL)

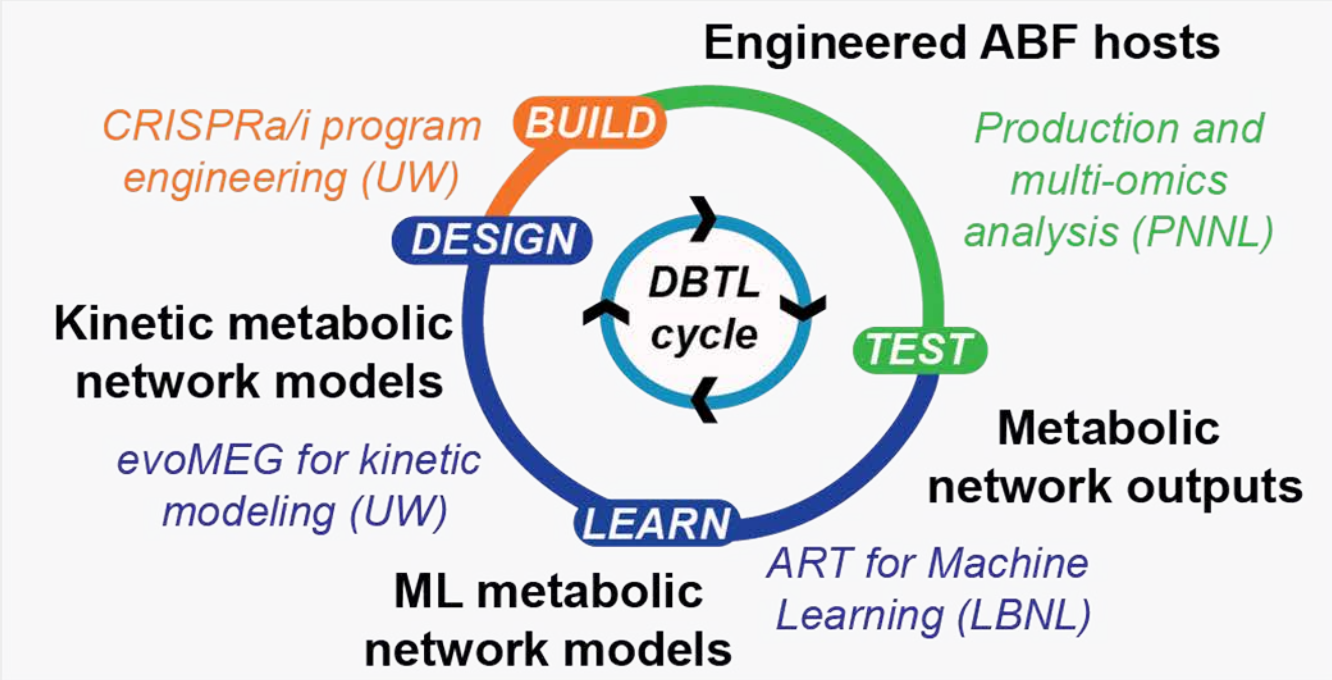
Enables:
- Accelerated data- and model-driven DBTL cycles

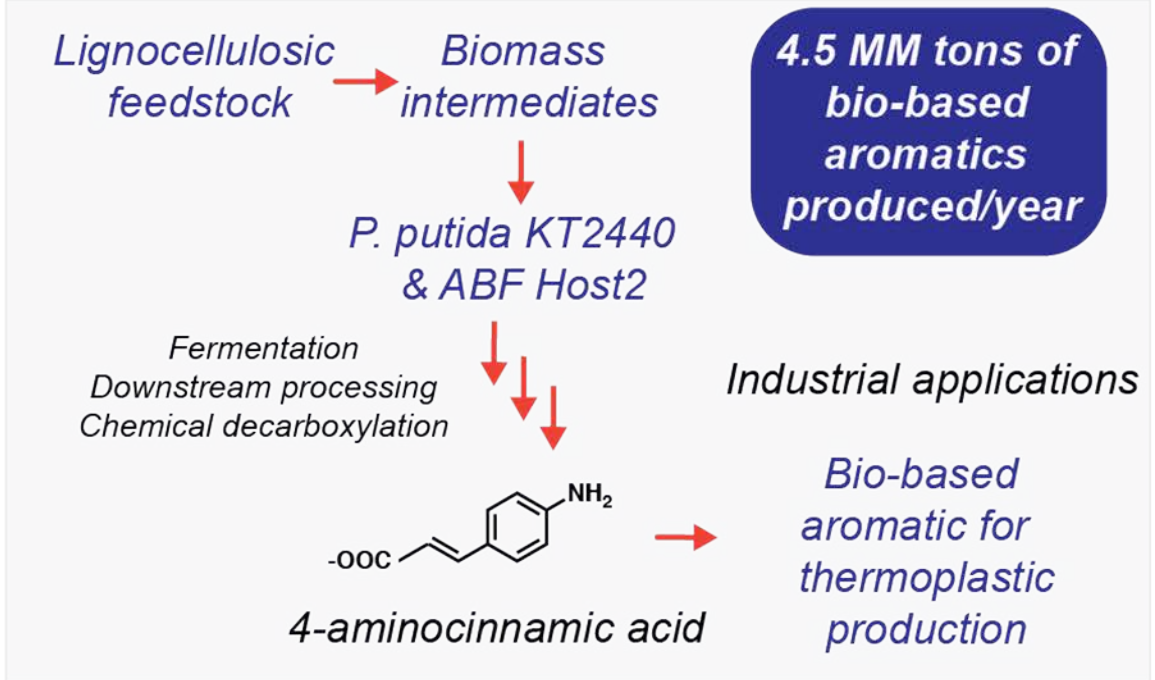
Goals:
- CRISPRa tools for 2 ABF organisms
- 30% increase in DBTL efficiency
- 2 ABF hosts engineered to produce an industrial bio-based aromatic

Pacific Northwest NATIONAL LABORATORY

BERKELEY LAB

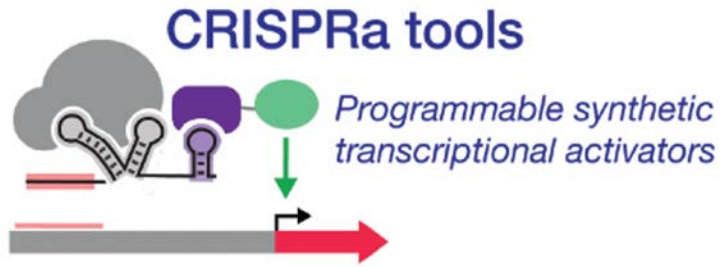
Project Overview: CRISPRa/i-enabled DBTL cycles to optimize bio-based aromatic production



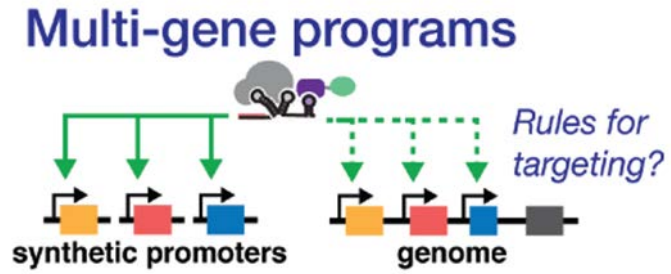


Project Overview: End of project goals

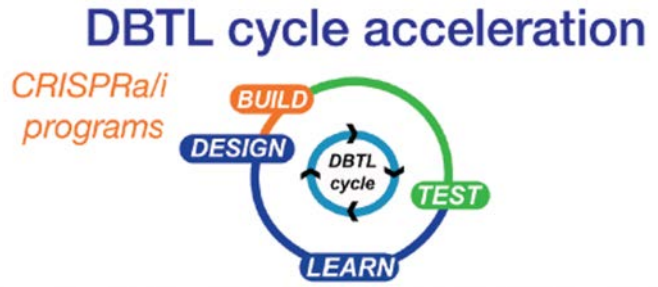
1. Develop and validate a strategy that will yield essentially an unlimited supply of orthogonal synthetic promoters for targeted CRISPRa in ABF hosts.



2. Demonstrate that complex multi-gene CRISPRa programs can be encoded as sets of easy-to-build, genetically-compact guide RNA programs.



3. Create and validate entirely new workflows for integrating CRISPRa/i program engineering with ABF DBTL cycles to rapidly optimize semi-synthetic 4-ACA production.

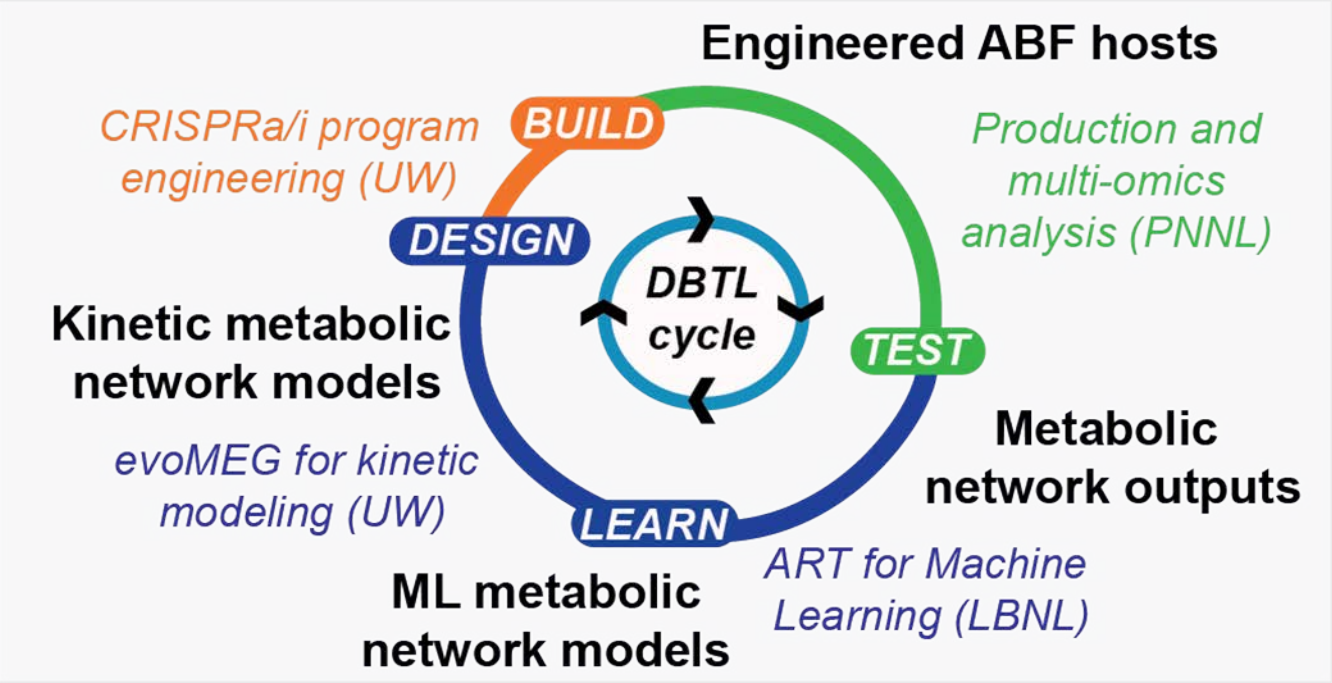


History: BETO site visit March 2020. Coordinated task work following COVID-19 shutdowns began July 2020.

1 – Management

I - Management: Project structure, team responsibilities and coordination strategy

Project structure and team responsibilities



Coordination strategy

Virtual meetings: Biweekly video calls

Updates: Biweekly updates by task leads with monthly tracking

Team Leads: Experts in synthetic biology and metabolic engineering, microbial strain development, multi-omics analysis, systems biology, machine learning, and computational biology

Project interfacing: *ad hoc* meetings with ABF members

Software: Team Google Site, Experimental Data Depot, DIVA, ART, evoMEG, Github

Team leads

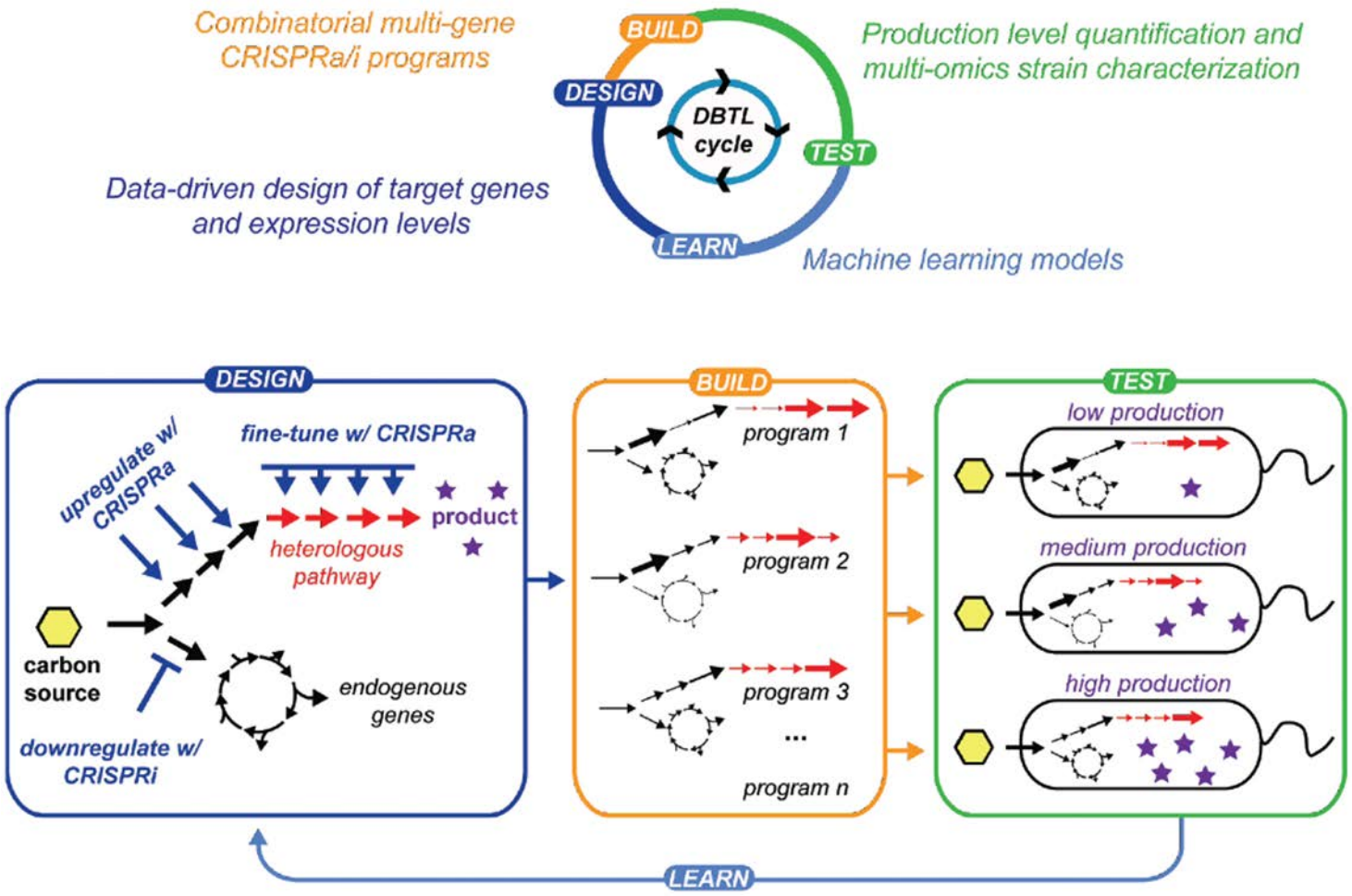
James Carothers (PI)
Jesse Zalatan
Herb Sauro

Rob Egbert
Alex Beliaev
Jon Magnusson (ABF)

Hector Garcia Martin (ABF)

2 – Approach

2 - Approach: CRISPRa/i-enabled DBTL cycles



Fontana, Sparkman-Yager, et al. *Curr. Opin Biotechnol.* 2020.

Integrate CRISPRa/i engineering with data- and model-driven workflows to rapidly optimize chemical production.

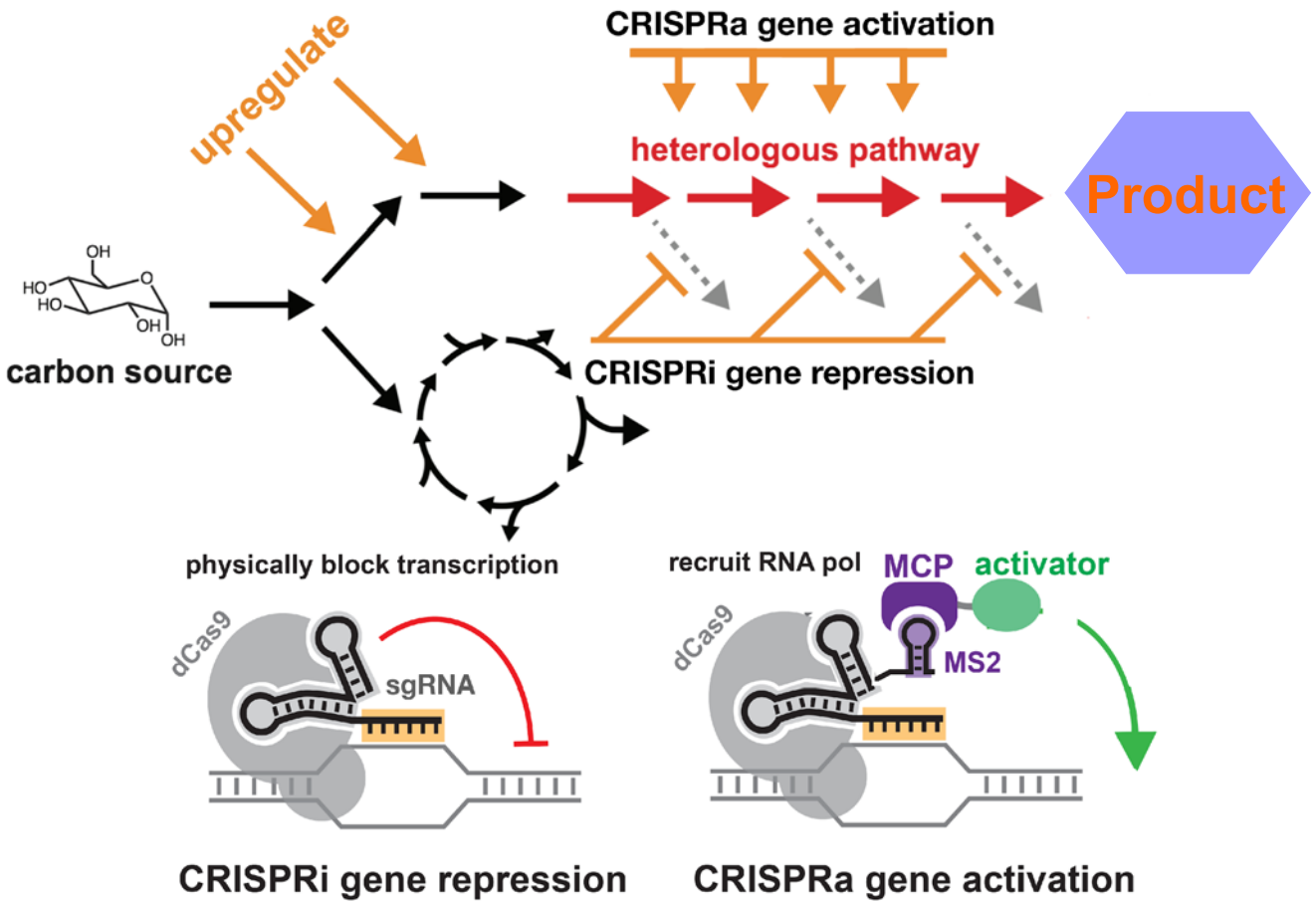
2 - Approach: Background on CRISPRa/i transcriptional programs in bacteria

CRISPR-Cas programs employ:
- Nuclease defective Cas9 (dCas9)
- Small guide RNAs (sgRNAs) specifying DNA target sites

CRISPRi gene repression:
- Target the dCas9 complex to promoter or open reading frame

CRISPRa gene activation:
- Modified guide RNAs (scaffold RNAs, scRNAs) with MS2 hairpin recruit a transcriptional activator (fused to MCP) to a promoter

Multi-gene CRISPRa/i programs:
- Simultaneous expression of multiple sgRNAs and scRNAs



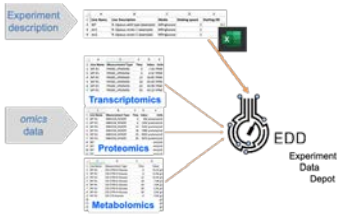
Dong, Fontana, et al. *Nature Comm.* 2018.
Fontana, Dong et al. *Nature Comm.* 2020.

Combine multi-gene CRISPR program engineering with new workflows to rapidly optimize bioproduction.

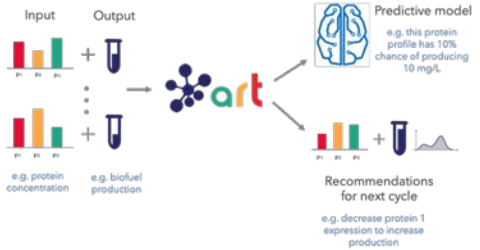
2 - Approach: Key challenges and technical approaches for achieving goals

- **Challenge:** CRISPRa in non-model bacteria is at an early stage of development
  Approach: Systematically define rules for effective CRISPRa to generalize synthetic gene regulation in ABF hosts

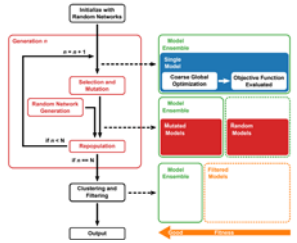
- **Challenge:** Outputs of multi-gene programs are difficult to predict
  Approach: Develop workflows to characterize outputs with multi-omic analysis



- **Challenge:** Combinatorial space of possible multi-gene programs is vast
  Approach: Apply ART to generate Machine Learning-derived recommendations and drive CRISPRa/i program design



- **Challenge:** ML-derived predictions can be difficult to rationalize
  Approach: Develop mechanistic modelling approaches with evoMEG to explain ML model prediction failures and improve ML-driven DBTL



Pacific Northwest NATIONAL LABORATORY

BERKELEY LAB

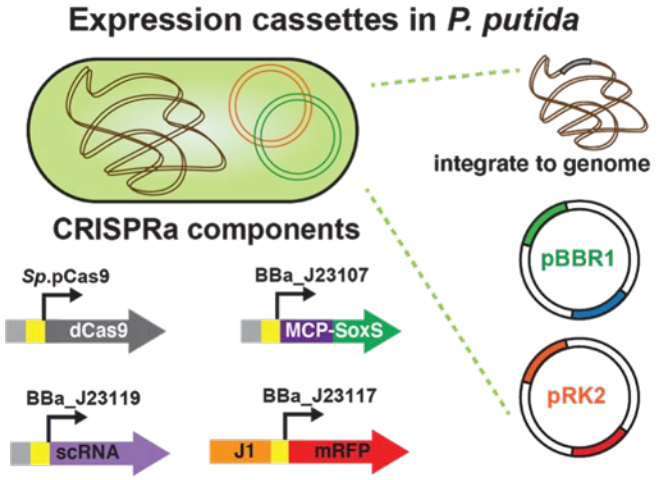
2 - Approach: Go/No-Go points

Go/No-Go

- Meet DOE requirements for verifying readiness of CRISPRa for activating gene expression in *P. putida* KT2440 >5-fold, and present computational tools for machine learning, and kinetic modeling.

Why it is critical to the project:

- Shows that tools for effective CRISPRa developed in *E. coli* can be ported to ABF bacterial hosts, and that the research infrastructure needed to attain the project research goals is in place.

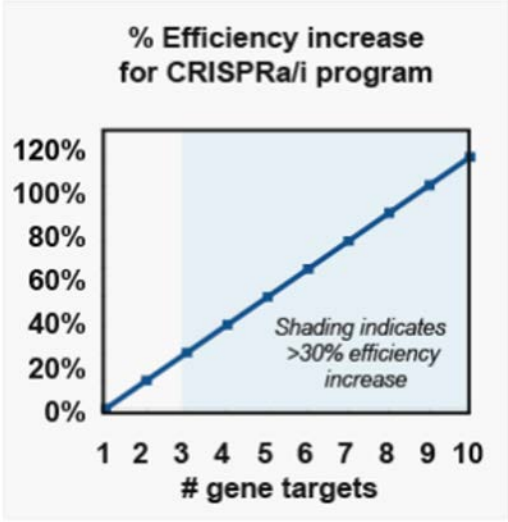


Go/No-Go

- Successful demonstration of 5 day/5 gene CRISPRa/i expression program build events in *P. putida* KT2440, corresponding to a >30% increase in DBTL cycle efficiency compared to 5 x 1-gene programming events.

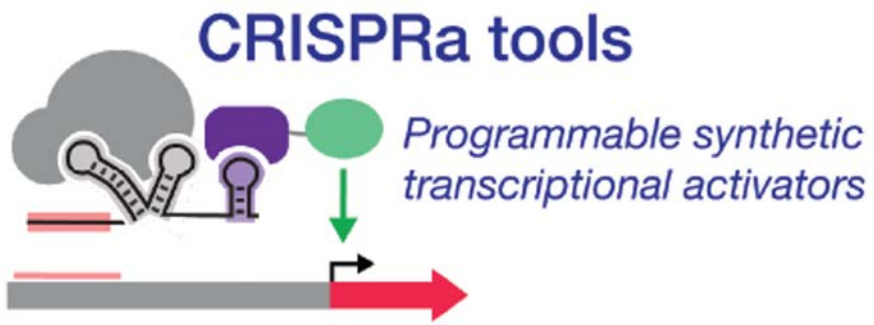
Why it is critical to the project

- Demonstrates feasibility of CRISPRa/i-enabled DBTL cycles to accelerate strain optimization compared to the state-of-the-art.



2 - Approach: Technical metrics used to measure progress

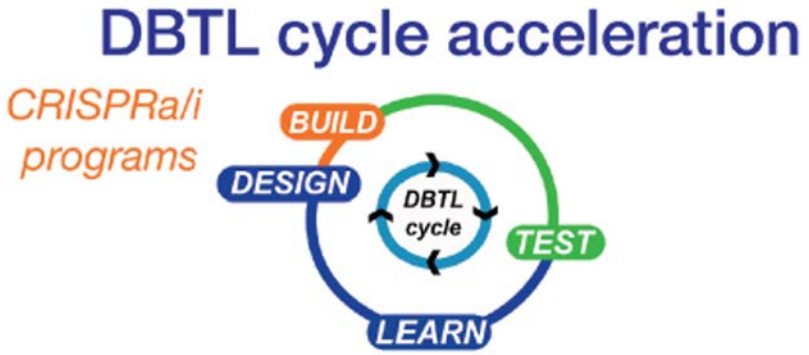
Quantifying CRISPRa-directed activation



CRISPRa activation
Fold-change in gene expression =

$$\frac{\textit{Level of GOI with On-target scRNA}}{\textit{Level of GOI with Off-target scRNA}}$$

Quantifying DBTL cycle efficiency



DBTL efficiency =
production titer per cycle per time

On a time basis with constant production titer increase per cycle, DBTL process efficiency can be calculated as:

$$\textit{Efficiency \%} = \frac{\textit{Time}_{\textit{Value-added}}}{\textit{Time}_{\textit{DTL + DNA assembly}}} \times 100 \qquad \textit{(eq. 1)}$$

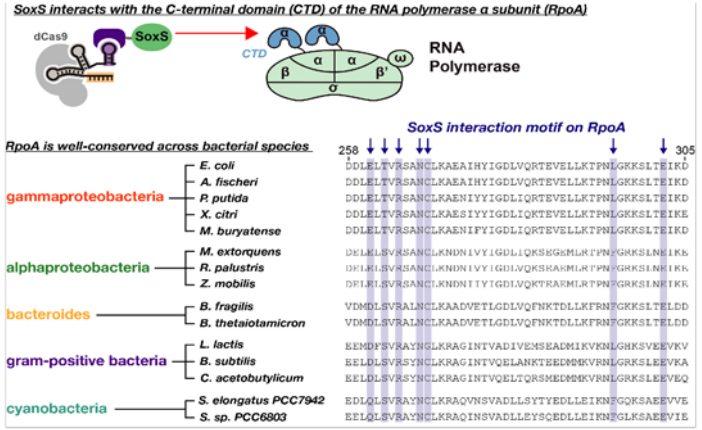
$$\textit{Time}_{\textit{Value-added}} = \textit{Time}_{\textit{DTL + DNA assembly}} \; \textit{Time}_{\textit{Strain build}} \qquad \textit{(eq. 2)}$$

3 – Impact

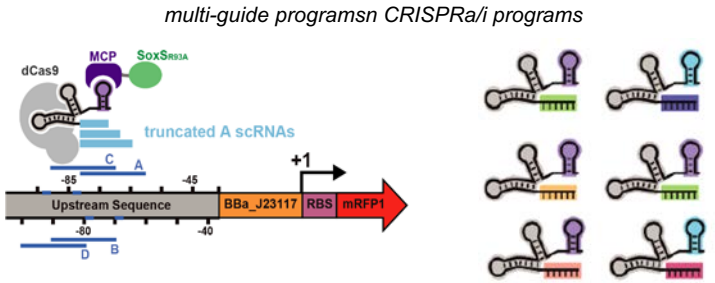
3 - Impact: CRISPRa/i-enabled DBTL cycles to optimize bio-based production

Impact on State of Technology

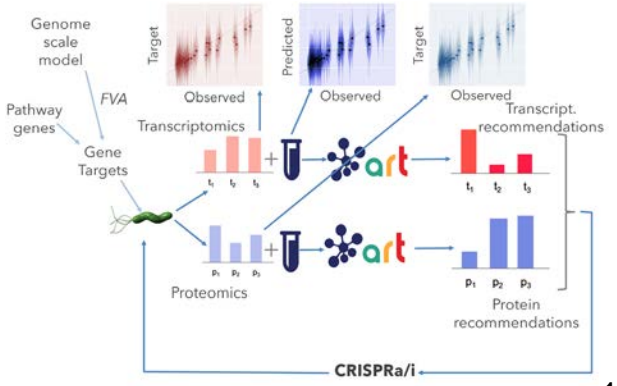
- Strategies for generalizing synthetic gene regulation in non-model bacteria with properties useful for bioproduction
  Deliverable: 5 orthogonal, tunable synthetic promoters and cognate scRNAs for programming the expression of heterologous genes in two ABF hosts



- Genetic technologies to create multi-gene CRISPRa/i programs
  Deliverable: Modular genetic constructs for expressing 5 gene CRISPRa/i programs in two hosts.



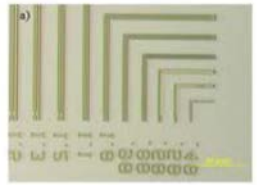
- Improved data- and model-driven microbial engineering
  Deliverable: Workflows for integrating machine learning and kinetic modeling to drive CRISPRa/i program engineering.



3 - Impact: CRISPRa/i-enabled DBTL cycles to optimize bio-based production

Impact on State of Technology cont'd

- Accelerated DBTL cycles
  Deliverable: DBTL efficiency increased >30% compared to the current state of the art.
- Microbial platform for bio-based aromatic production
  Deliverable: *P. putida* KT2440 strain engineered to produce 4-aminocinnamic acid (4-ACA) in bioreactor conditions from biomass intermediates.



Photoresists for nanofabrication

Polymers for photovoltaic materials

Technology Transfer Activities

- Records of Invention leading to patent applications on new tools, engineered strains and new pathways in the pipeline
- Peer-reviewed publications describing foundational work in the pipeline
- Commercialization activities planned in collaboration with UW CoMotion (UW technology transfer office)

4 – Progress and Outcomes

4 - Progress and Outcomes: Progress according to the Project Management Plan

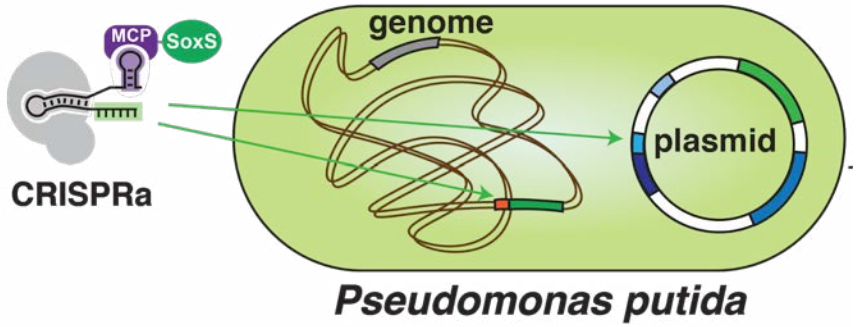
Task	Subtask	Due	% Complete
1: Initial Project Verification (UW, LBNL, PNNL)	Successfully pass DOE pre-project verification requirements	4/20	100%
2: Implement single-gene CRISPRa/i perturbations in *P. putida* KT2440 to train Machine Learning (ML) models (UW, PNNL, LBNL)	Develop CRISPRa tools for single gene perturbations in *P. putida* KT2440	1/21	100%
	Engineer single gene perturbations using CRISPRa/i	4/21	55%
	Generate a predictive model of production by combining ML and genome-scale models	7/21	3%
3: Kinetic model-driven design of multi-gene CRISPRa/i programs in *P. putida* KT2440 (UW)	Engineer multi-gene CRISPRa/i expression programs in *P. putida* KT2440	4/21	18%
	Train the kinetic model using universal or lin-log rate laws and perturbation experiments.	4/21	25%
	Validate the kinetic model using CRISPRa/i- directed perturbations.	1/22	0%
4: Apply accelerated DBTL cycles to optimize bio-based aromatic production in P*. putida* KT2440 (UW, PNNL, LBNL)	Apply DBTL cycles to optimize 4-aminocinnamic acid production with multi-gene CRISPRa/i programs.	10/21	0%
	Evaluate *P. putida* KT2440 4-aminocinnamic acid production in 2 L bioreactors.	10/21	0%
Task 5: Develop CRISPRa in multiple ABF hosts (UW)	Pilot CRISPRa in four ABF hosts with conserved RpoA interaction motifs	1/22	12.5
	Develop tools for CRISPRa in a second ABF host (ABF Host2)	4/22	0%
6: Apply accelerated DBTL cycles to optimize bio-based aromatic production in ABF Host2 (UW, PNNL, LBNL)	Apply Transfer Learning to generate a ABF Host2 metabolic network model	10/22	0%
	Calibrate kinetic models of ABF Host2 metabolism	10/22	0%
	Apply DBTL cycles to optimize 4-aminocinnamic acid production with multi-gene CRISPRa/i programs	1/23	0%

(% complete as of 1/21)

4 - Progress and Outcomes: Most important technical accomplishments achieved

Most important technical accomplishments achieved

- Validated an experimental workflow to port CRISPRa to novel bacteria
- Developed genetic tools for reliable CRISPRa-directed expression from synthetic promoters
- Achieved CRISPRa-directed activation from endogenous promoters



End of Project Goals

Goal 1. Develop and validate a strategy that will yield essentially an unlimited supply of orthogonal synthetic promoters for targeted CRISPRa in ABF hosts.

Goal 2. Demonstrate that complex multi-gene CRISPRa programs can be encoded as sets of easy-to-build, genetically-compact guide RNA programs.

Goal 3. Create and validate entirely new workflows for integrating CRISPRa/i program engineering with ABF DBTL cycles to rapidly optimize semi-synthetic 4-ACA production.

Successfully ported bacterial CRISPRa to *P. putida* KT2440

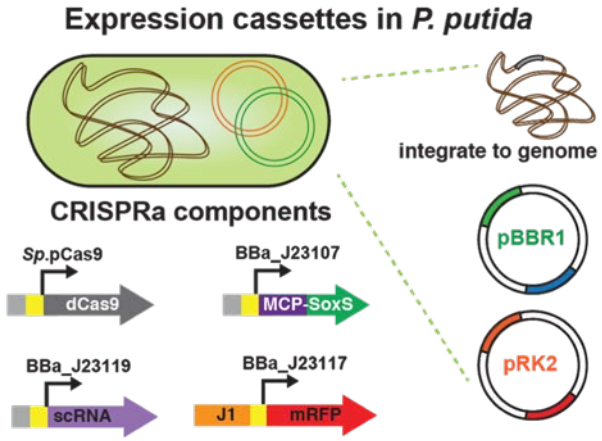
4 - Progress and Outcomes: Workflow to port CRISPRa to novel bacteria

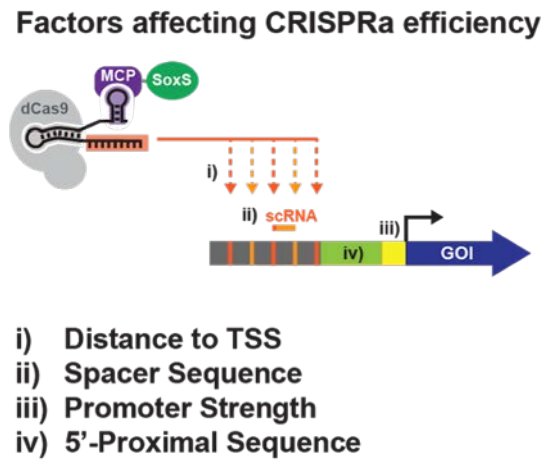
Challenge

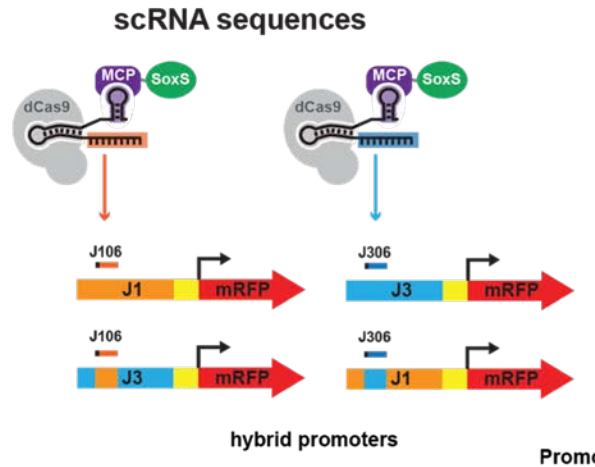
- The development of bacterial CRISPRa has lagged due to the lack of effective transcriptional activators and the complexity of rules governing CRISPRa-directed transcriptional activation.

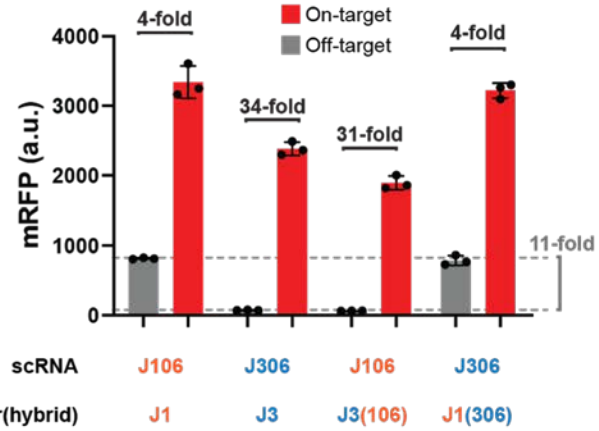
Outcomes

- Constructed genetic components and established experimental approaches to permit CRISPRa machinery developed in *E. coli* to be expressed and utilized in *P. putida*.
- Experimentally investigated promoter features impacting CRISPRa to identify designs permitting high levels of heterologous gene expression.









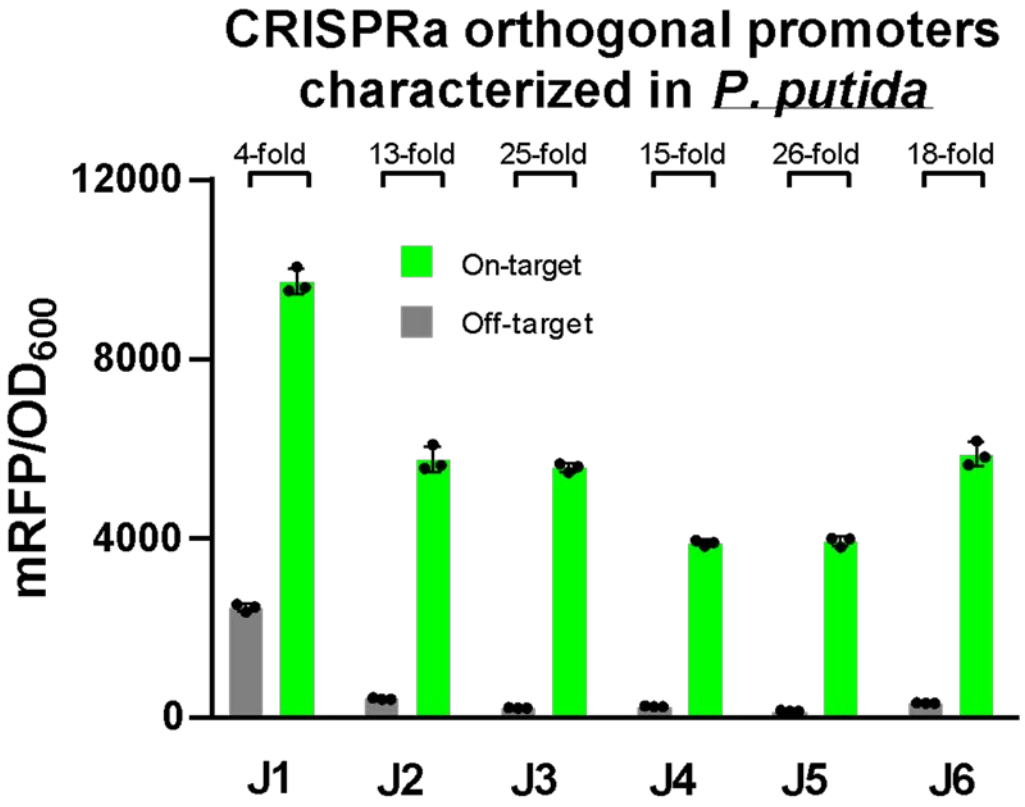
4 - Progress and Outcomes: Genetic tools for reliable CRISPRa from synthetic promoters

Q2 SMART Milestone 2.1.1 (Task 2)

- Validation of 5 orthogonal synthetic promoters and cognate scRNA pairs that can be used to program 10-fold CRISPRa activation of heterologous genes in *P. putida* KT2440

Outcomes

- 6 synthetic CRISPRa promoters and guide RNA (scRNA) pairs were designed, constructed and tested in *P. putida* KT2440.
- 5 synthetic promoters gave high levels of activation (>>5-fold) compared to the off-target negative control.

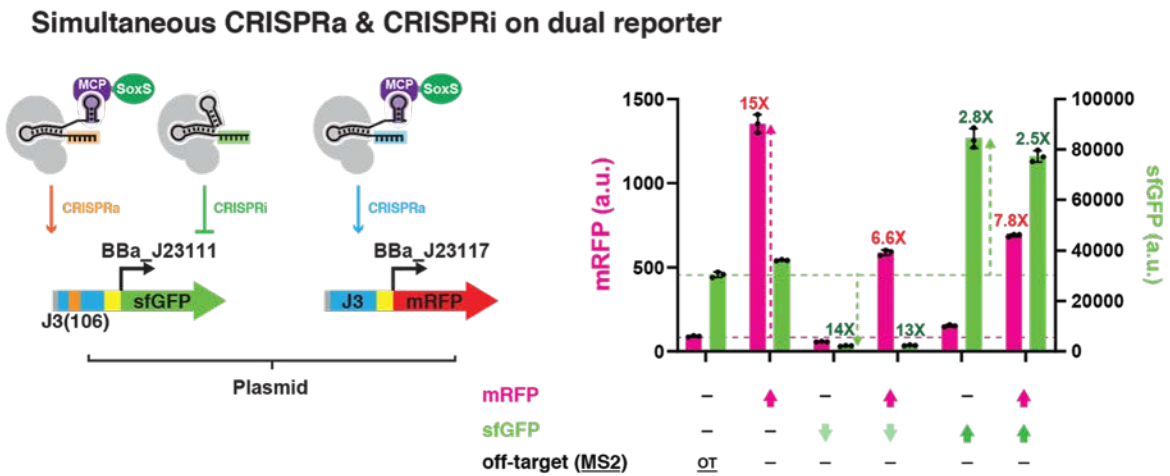


"5 promoters should give >5-fold CRISPRa compared to off-target control"

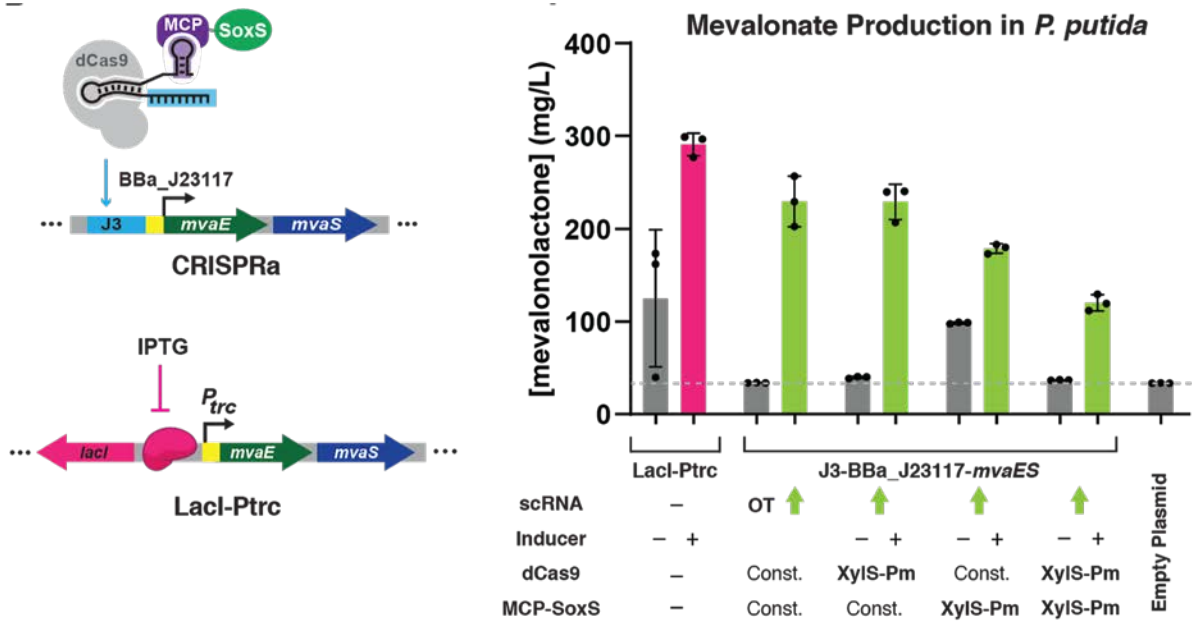
Strategy will yield an unlimited supply of orthogonal synthetic promoters for CRISPRa (EOP Goal 1).

4 - Progress and Outcomes: Genetic tools for reliable CRISPRa from synthetic promoters in *P. putida* KT2440

Multi-gene CRISPRa/CRISPRi



Inducible CRISPRa directed bioproduction



Creating complex multi-gene CRISPRa programs encoded as genetically-compact guide RNA programs (EOP Goal 2).

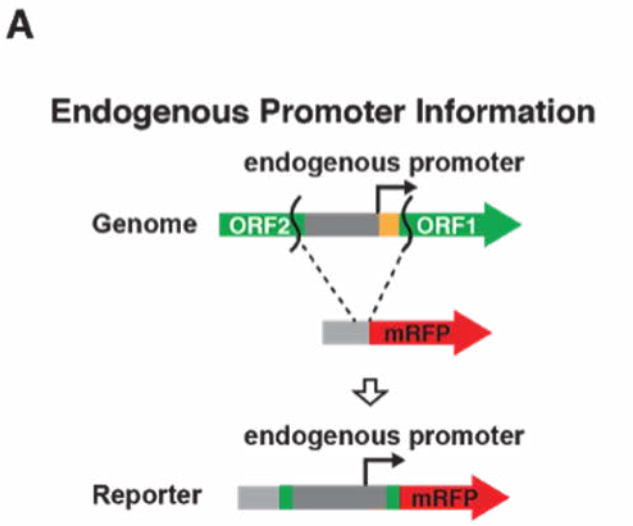
4 - Progress and Outcomes: CRISPRa from endogenous promoters

Q4 Milestone 2.1.3 (Task 2)

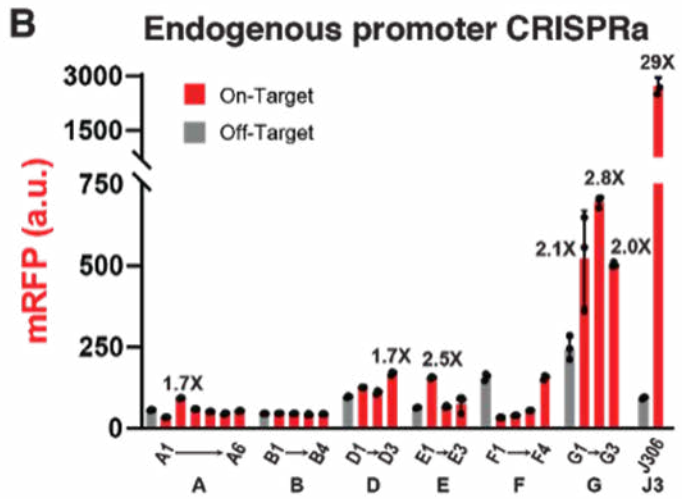
- 1.5-2 fold CRISPRa from at least 2 arbitrary endogenous genes in *P. putida* KT2440.

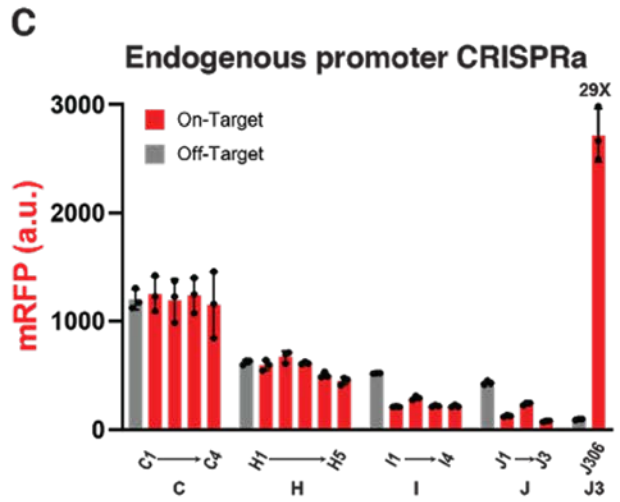
Outcomes

- Putative promoter sequences between two open reading frames (ORFs) with 60 bp flanking sequences were incorporated into the mRFP reporter. scRNAs were introduced for all potentially activatable target sites predicted to be effective for activation.



	Name	Gene/Protein
A:	PP_1776	mannose-6-phosephate isomerase
B:	PP_4812	3-methyladenine DNA glycosylase
C:	PP_3839	*adhP*
D:	PP_1992	*asd*
E:	PP_0786	*trxB*
F:	PP_1972	*tyrB*
G:	PP_3668	*katG*
H:	PP_5046	*glnA*
I:	PP_1231	*nadA*
J:	PP_4701	*pgi-II*





Creating complex multi-gene CRISPRa programs encoded as genetically-compact guide RNA programs (EOP Goal 2).

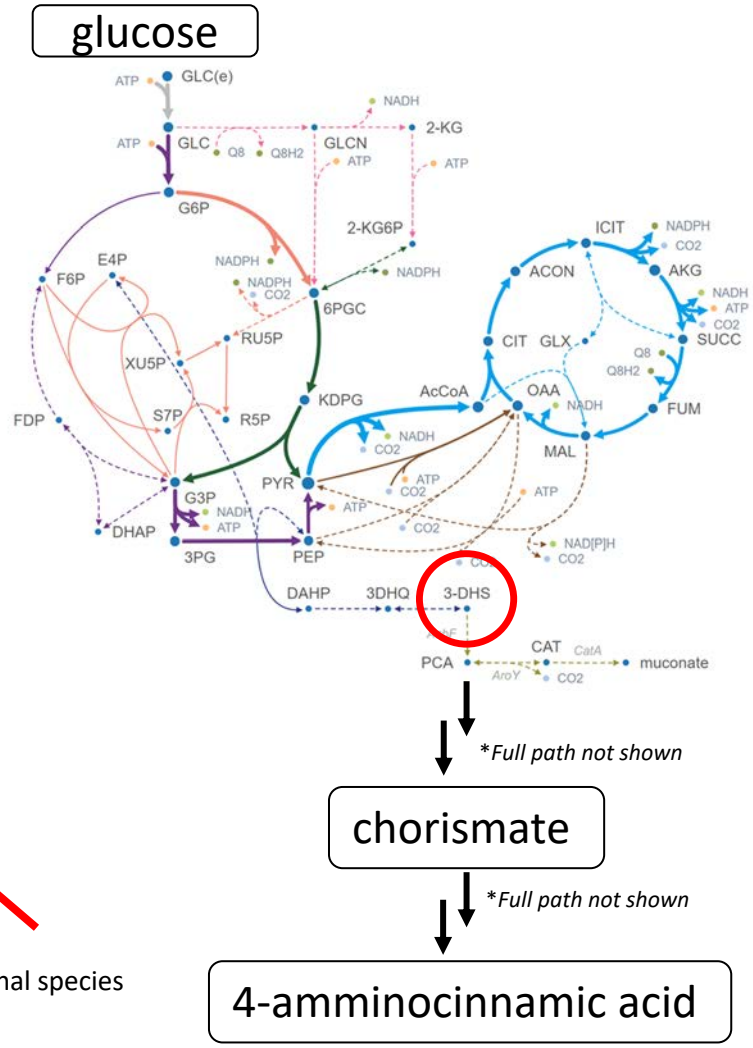
4 - Progress and Outcomes: Computational tools for CRISPRa/i enabled DBTL cycles

Q3 Milestone 3.2.2 (Task 3)

- Train the kinetic model using universal, and lin-log rate laws.

Outcomes

- Estimated initial rate values and elasticities from limited *P. putida* model. Estimated initial [metabolite] by finding correlation between [metabolite] and elasticities in *E. coli* model. Model is used to compute initial set of pathway targets.



- Pathway up to 4-amminocinnamic acid
- Estimating rates using the linlog equation

$$\chi = \log\frac{x}{x^*};\ \gamma = \log\frac{y}{y^*};\ \hat{v} = \frac{v}{v^*};\ \hat{e} = \frac{e}{e^*}$$

$$v = diag(v^*\ \hat{e})(1_n + \epsilon_x^*\chi + \epsilon_y^*\gamma)$$

Total rate for each reaction (normalized)

Rate of enzymes

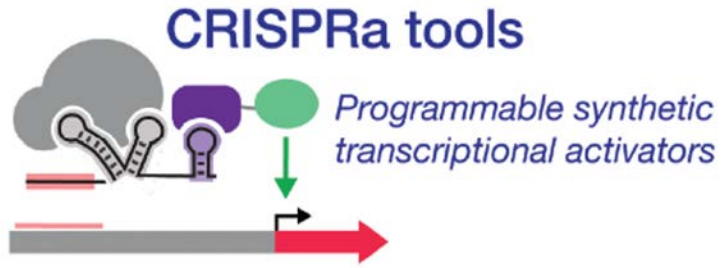
Internal species

External species

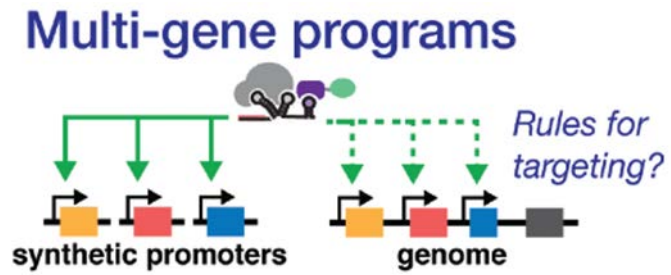
Creating workflows for integrating CRISPRa/i program engineering with ABF DBTL cycles (EOP Goal 3).

Summary

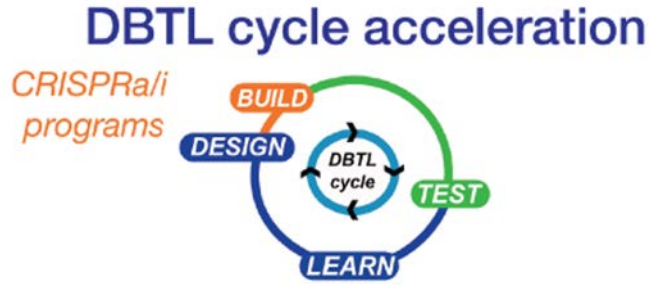
1. Developing and validating a strategy that will yield essentially an unlimited supply of orthogonal synthetic promoters for targeted CRISPRa in ABF hosts.



2. Creating approaches to build complex multi-gene CRISPRa programs that can be encoded as sets of easy-to-build, genetically-compact guide RNA programs.



3. Creating and validating entirely new workflows for integrating CRISPRa/i program engineering with ABF DBTL cycles to rapidly optimize semi-synthetic 4-ACA production.



Engineering multi-gene CRISPRa/i programs to accelerate DBTL cycles in ABF Hosts

Project Team Members

University of Washington
James Carothers (PI)
Jesse Zalatan (Co-PI)
Herb Sauro (Co-PI)
Ava Karanjia
Cholpisit Ice Kiattisewee
David Sparkman-Yager
Joely Nelson
Ian Faulkner
Neel Shah
Janis Shin
Jason Fontana

Pacific Northwest National Laboratory
Rob Egbert (Co-PI)
Alex Beliaev (Co-PI)
Jon Magnusson (ABF)(Co-PI)
Jeremy Zucker
Kristin Burnum Johnson
Nathalie Munoz

Lawrence Berkeley National Laboratory
Hector Garcia Martin (ABF)(Co-PI)
Tijana Radivojevic

Quad Chart Overview

Timeline

- 1/1/2020
- 1/1/2023

	FY20 Costed	Total Award
DOE Funding	(10/01/2019 – 9/30/2020) *$221K*	(negotiated total federal share) *$1.83M*
Project Cost Share	$55.3K	$0.46M

Project Goal

The goal of this project is to develop technologies and workflows to combine multi-gene CRISPRa/i program engineering with computational modeling, machine learning, and multi-omics data to enhance the efficiency of design-build-test-learn (DBTL) cycles for optimizing bioproduction in ABF hosts.

End of Project Milestone

>30% increase in DBTL cycle efficiency compared to 5 x 1 gene programming events.

Project Partners*

- Pacific Northwest National Laboratory
- Lawrence Berkeley National Laboratory

Funding Mechanism

FOA Number: DE-FOA-0002029,
Topic Area: AOI 7b: Agile BioFoundry
Year: 2019

*Only fill out if applicable.

Additional Slides

Responses to Previous Reviewers' Comments

- If your project has been peer reviewed previously, address 1-3 significant questions/criticisms from the previous reviewers' comments which you have since addressed
- Also provide highlights from any Go/No-Go Review

The project has not been previously peer reviewed and no highlights from the verification Go/No-Go to report.

Publications, Patents, Presentations, Awards, and Commercialization

Publications:

Kiattisewee, C., Dong, C., Fontana, J., Sugianto, W., Peralta-Yahya, P., Carothers, J.M.*, Zalatan, J.G.* "Portable bacterial CRISPR transcriptional activation enables metabolic engineering in *Pseudomonas putida*". *Submitted to Metabolic Engineering. In revision.*

Patents:

UW Record of Invention related to CRISPRa tools in *P. putida* KT2240 for metabolic engineering applications. *In Preparation.*

Presentations:

"Tool Development for Pathway Engineering", *AIChE National Meeting*. Presented by James M. Carothers, November 18, 2020.

Commercialization efforts:

Commercialization activities planned in collaboration with UW CoMotion (UW technology transfer office)

History: BETO site visit March 2020. Coordinated task work following COVID-19 shutdowns began July 2020.