DOE Bioenergy Technologies Office (BETO) 2023 Project Peer Review

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

Tuesday, April 4, 2023
Technical Session Review Area: Agile BioFoundry Consortium

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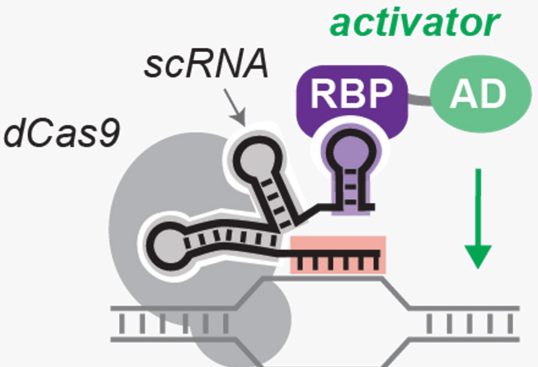
Project Overview

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

1. CRISPRa tools

CRISPRa (Activation)

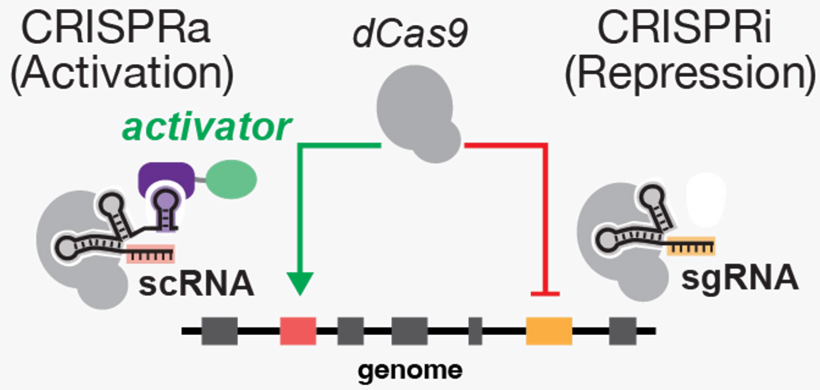


modified guide RNA (scRNA) recruits activation domain

ABF host	CRISPRa status
P. putida	Demonstrated
A. baylyi	Demonstrated

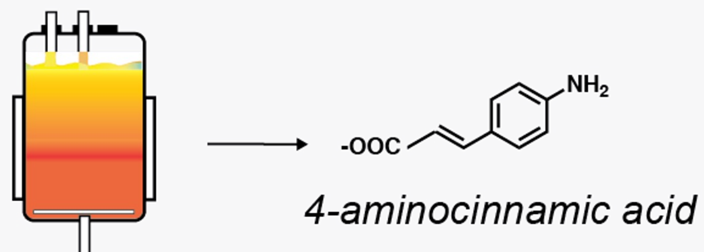
2. Transcriptional programs

Multi-Gene Programs



scRNA expression directs CRISPRa
sgRNA expression directs CRISPRi

3. Industrial aromatic production



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
- ABF host engineered to produce an industrial bio-based aromatic

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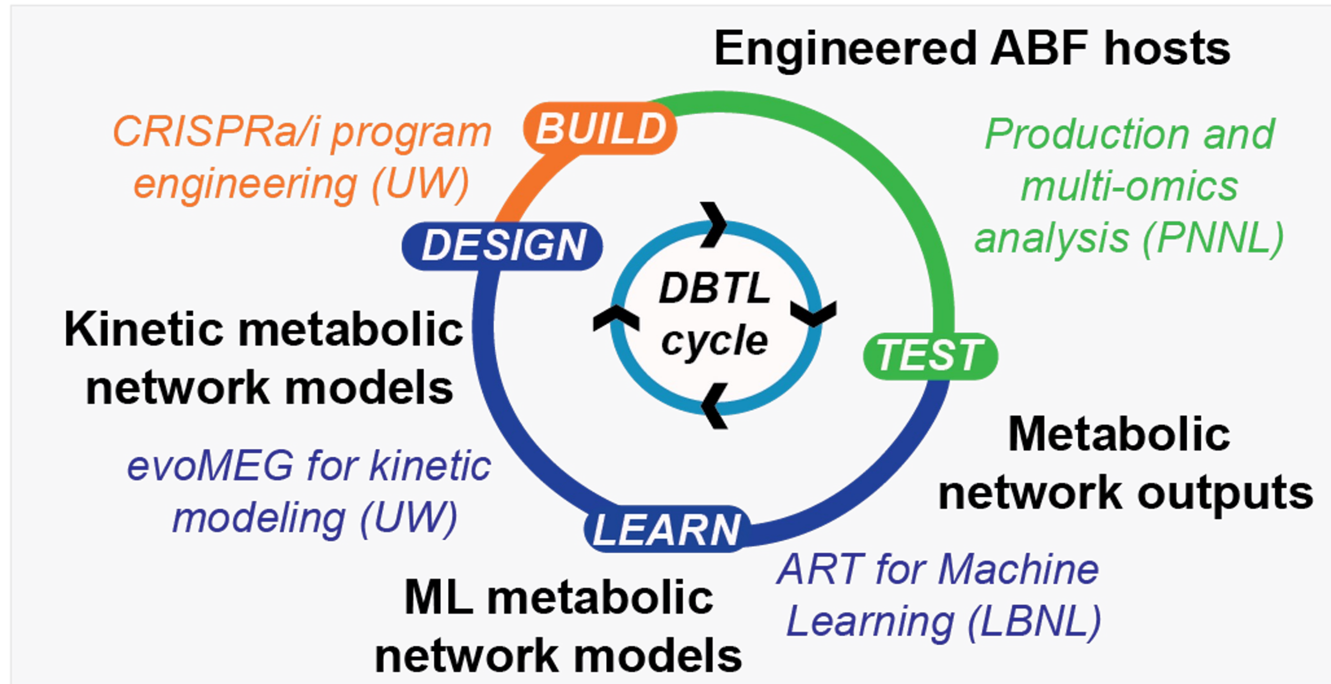
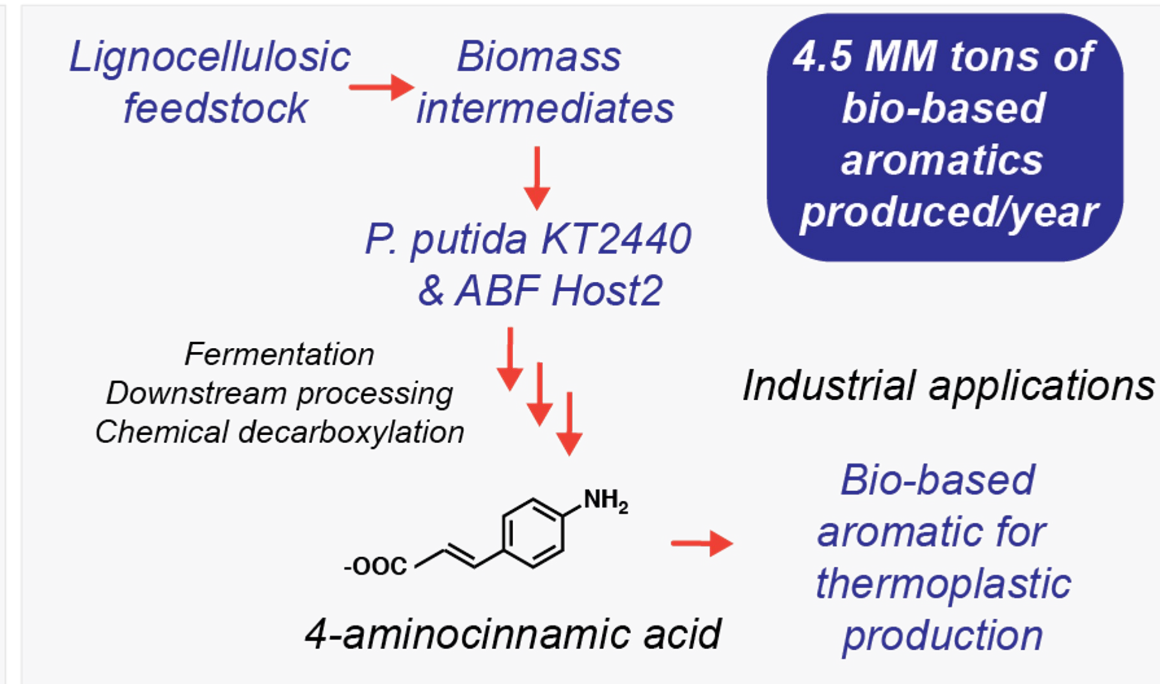
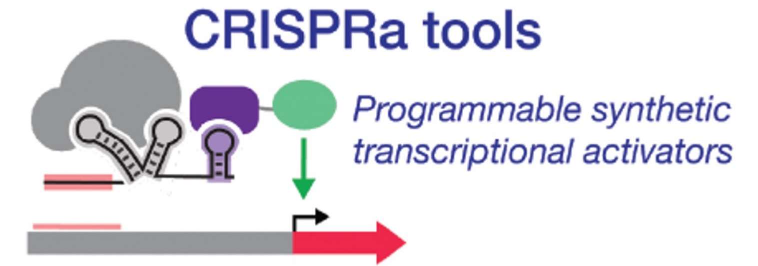
Project Overview: CRISPRa/i-enabled DBTL cycles to optimize bio-based aromatic production



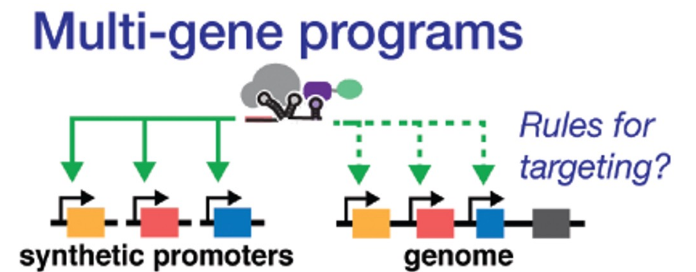


Project Overview: End of project goals

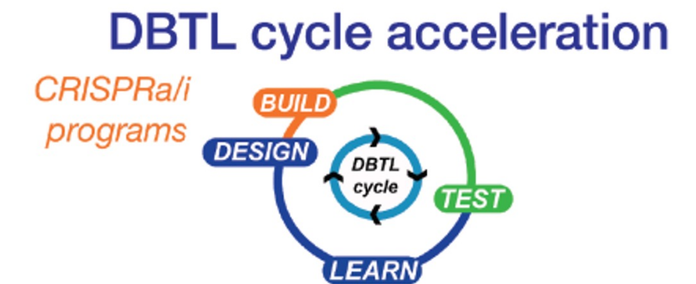
1. Develop and validate a strategy that will yield essentially an unlimited supply of orthogonal synthetic promoters for targeted CRISPRa in ABF hosts.



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: July 2020: Coordinated work began after COVID-19 shutdowns. Fall 2022: BP3 Intermediate Verification.

1 – Approach

I - 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.

I - 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:

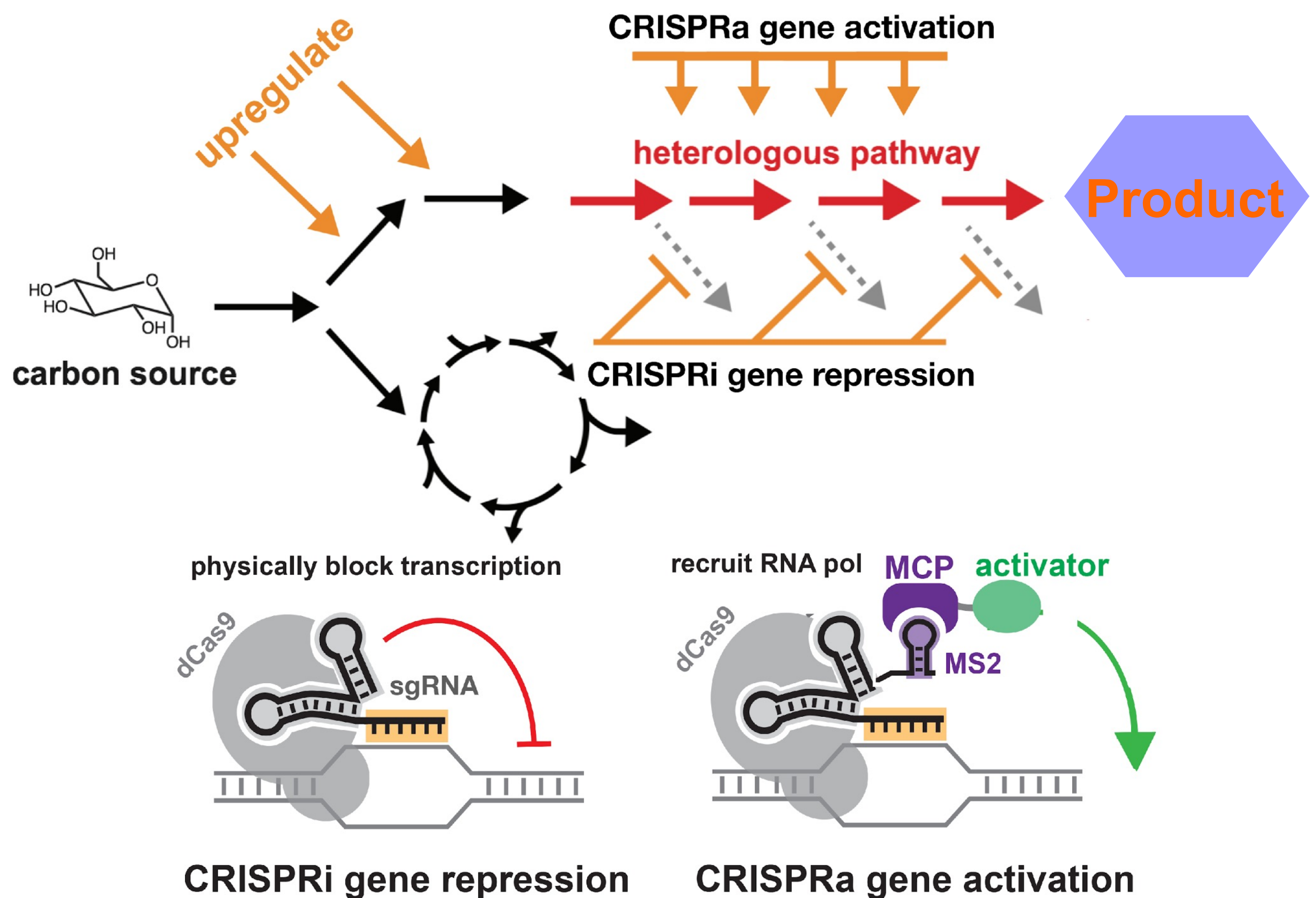
- Target the dCas9 complex to promoter or open reading frame

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

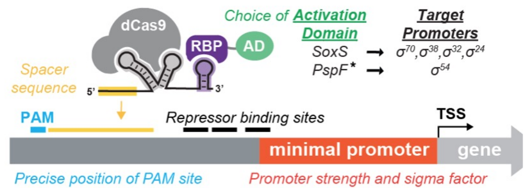


Dong, Fontana, et al. *Nature Comm.* 2018.
Fontana, Dong et al. *Nature Comm.* 2020.

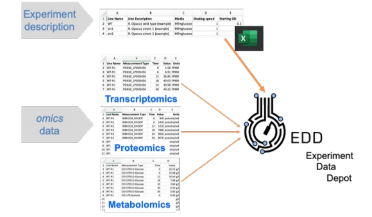
Combine multi-gene CRISPR program engineering with new workflows to rapidly optimize bioproduction.

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

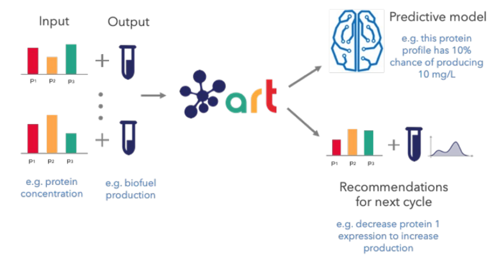
- **Challenge:** CRISPRa in non-model bacteria is at an early stage of development
  Approach: Systematically define rules for effective CRISPRa to generalize synthetic gene regulation in ABF hosts



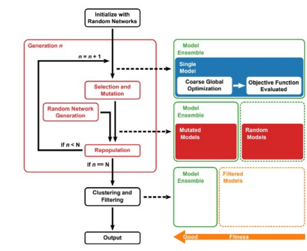
- **Challenge:** Outputs of multi-gene programs are difficult to predict
  Approach: Develop workflows to characterize outputs with multi-omic analysis



- **Challenge:** Combinatorial space of possible multi-gene programs is vast
  Approach: Apply ART (Radivojević et al. 2020. *Nature Comm.*) 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 to explain ML model prediction failures and improve ML-driven DBTL



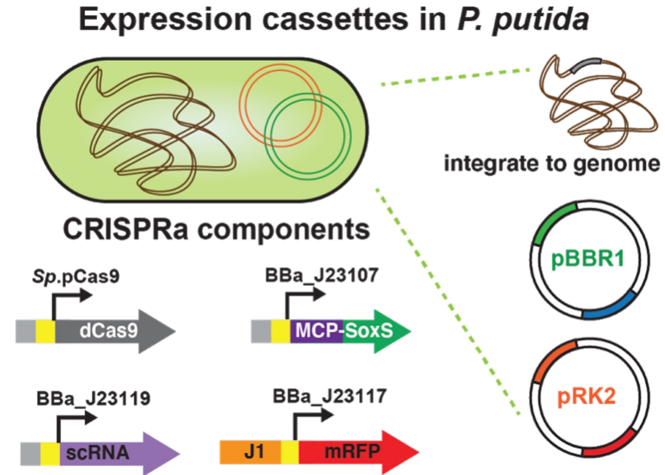
I - Approach: Go/No-Go points

Budget Period 1 Go/No-Go (Verification in Spring 2020)

- 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 was critical to the project:

- Showed 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 was in place.

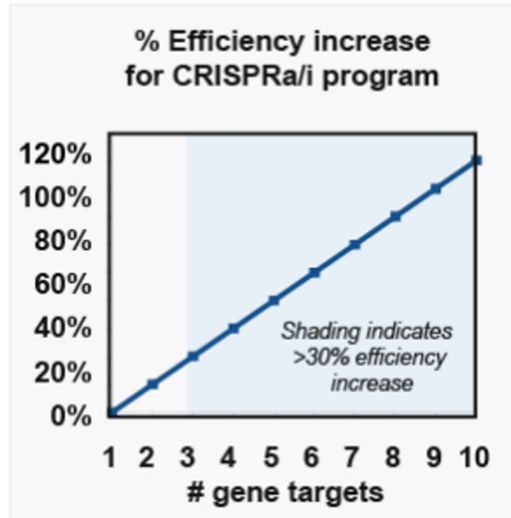


Budget period 2 Go/No-Go (Verification in Fall 2022)

- 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.

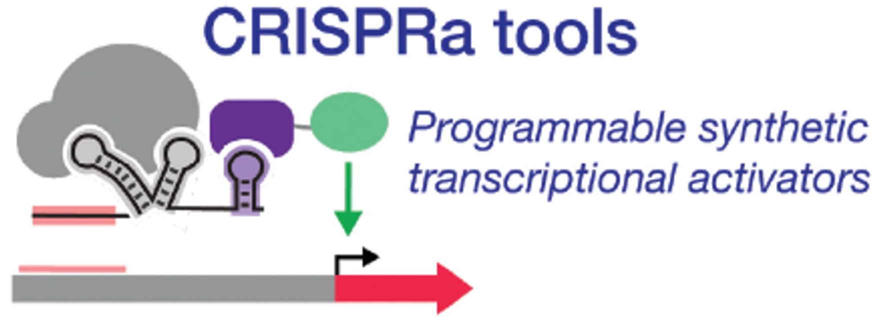
Why it was critical to the project

- Demonstrated feasibility of CRISPRa/i-enabled DBTL cycles to accelerate strain expression programming compared to the state-of-the-art.



I - Approach: Technical metrics used to measure progress

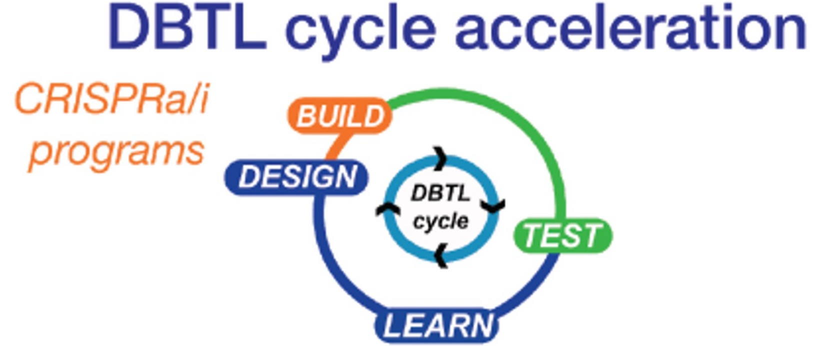
Quantifying CRISPRa-directed activation



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

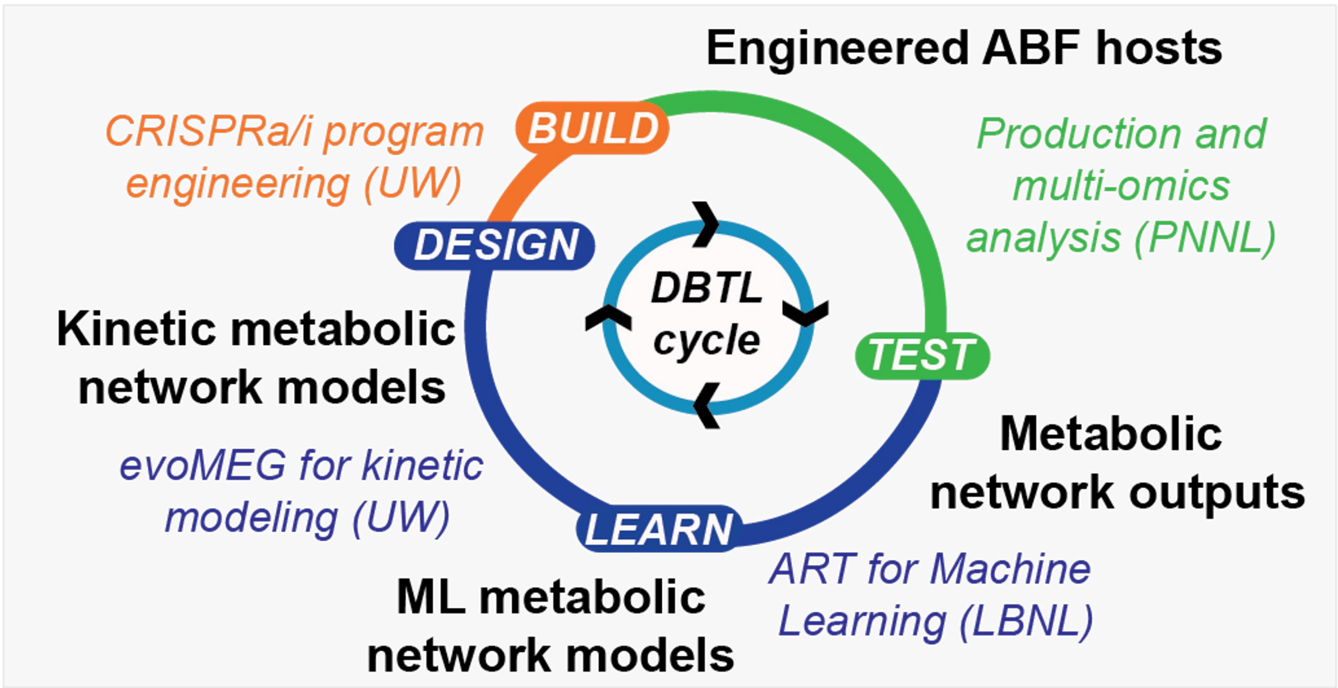
On a time basis with constant production titer increase per cycle, DBTL process efficiency can be calculated as:

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

$$Time_{Value\text{-}added} = Time_{DTL\ +\ DNA\ assembly} - Time_{Strain\ build} \qquad (eq.\ 2)$$

I - Approach: Management plan and implementation strategy

Project structure and team responsibilities



Coordination strategy for risk identification and mitigation

Virtual meetings: Bimonthly video calls

Updates: Bimonthly 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, ART, Github

Team leads

James Carothers (PI)
Jesse Zalatan
Herb Sauro

Alex Beliaev
Jeremy Zucker
Nathalie Munoz

Hector Garcia Martin (ABF)

2 – Progress and Outcomes

2 - 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	BP1	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	BP2	100%
	Engineer single gene perturbations using CRISPRa/i	BP2	100%
	Generate a predictive model of production by combining ML and genome-scale models	BP2	100%
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	BP2	100%
	Use ML models as proxies for metabolism to train kinetic models	BP2	100%
	Validate the kinetic model using CRISPRa/i- directed perturbations.	11/23	50%
4: Apply accelerated DBTL cycles to optimize bio-based aromatic production in P. *putida* KT2440 (UW, PNNL, LBNL)	Debottleneck pathway flux by operon refactoring	7/23	50%
	Apply DBTL cycles to optimize 4-aminocinnamic acid production with multi-gene CRISPRa/i programs.	11/23	0%
	Evaluate the efficacy of multi-guide CRISPRa/i programs and DBTL-based optimization by measuring *P. putida* KT2440 4-ACA production	1/24	20%
5: Develop CRISPRa in multiple ABF hosts (UW)	Pilot CRISPRa in ABF hosts with conserved RpoA interaction motifs	BP2	100%
	Develop tools for CRISPRa in a second ABF host (*A. baylyi*)	11/23	75%

(% complete as of 1/23), BP1 = Budget Period 1, BP2 = Budget Period 2
COVID-related delays were accomodated with 1-year no-cost extension

2 - Progress and Outcomes: Most important technical accomplishments achieved

P. putida **CRISPRa/i tool and 4-ACA production strain development (Task 2)**

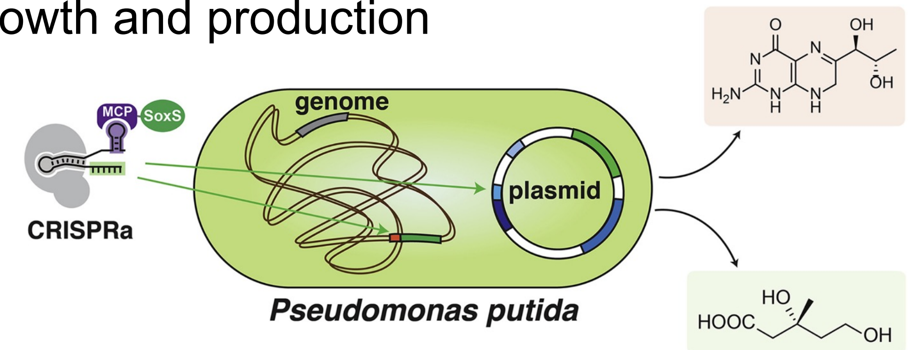
- Developed genetic tools for activating engineered and genomic promoters
- Achieved direct microbial bioproduction of 4-ACA from glucose

Multi-gene CRISPRa/i program engineering (Subtask 3.1/Milestone 4.2.3)

- Created approach for building genetically-compact, multi-guide programs
- Demonstrated 5-day build events of 6 guide programs, accelerating DBTL cycles >30%

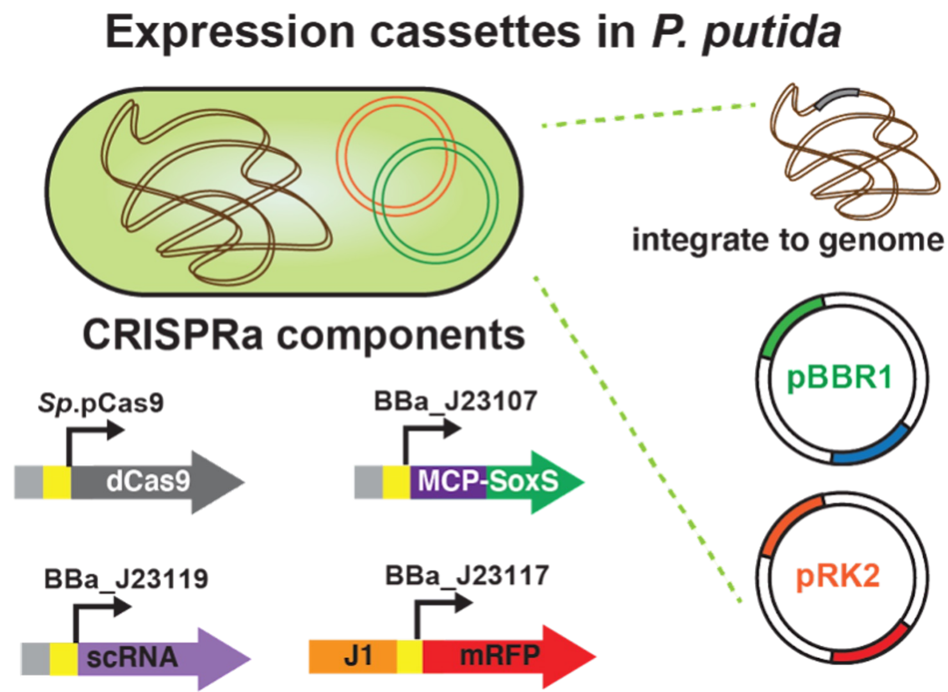
Model and data-driven DBTL cycle optimization (DBTL cycle 0)(Task 2/Task 4)

- CRISPRa/i targets selected using genome scale models
- Build 42 strains with multi-gene CRISPRa/i programs perturbing genomic targets
- DBTL cyle 0 CRISPRa/i perturbations generate variations in growth and production



Next: Apply accelerated DBTL cycles to optimize bio-based aromatic production in *P. putida* KT2440.

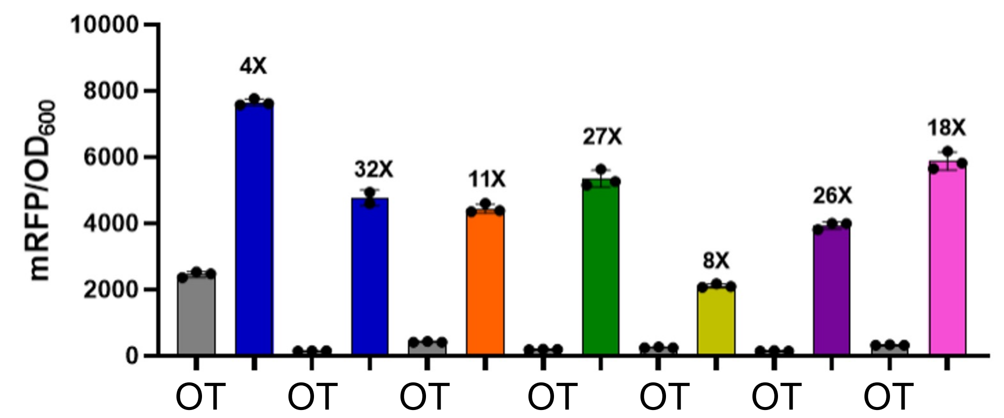
Porting tools for effective CRISPRa from *E. coli* to *P. putida* KT2440



Kiattisawee et al. *Metabolic Engineering* 2021.

CRISPRa of engineered promoters in *P. putida*

New toolbox of components for programming heterologous gene expression in ABF hosts.



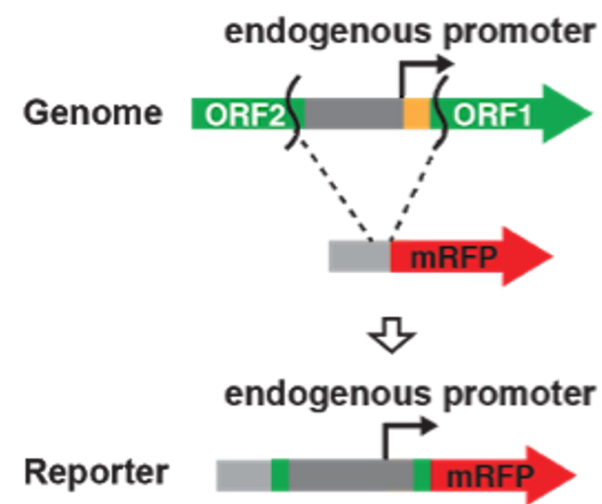
OT = Off target

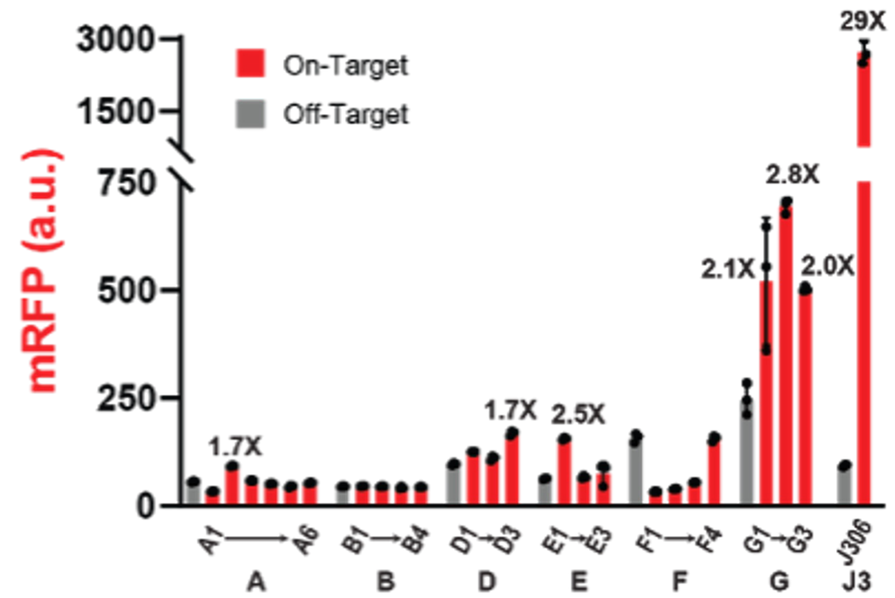
X-fold activation compared to OT indicated

Milestone 2.1.1: CRISPRa can be ported to new hosts by tuning component expression and characterizing rules.

2 - Progress and Outcomes: Task 2. *P. putida* CRISPRa/i tool development

CRISPRa of genomic promoters in *P. putida* KT2440





	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*

Kiattisawee et al. *Metabolic Engineering* 2021.

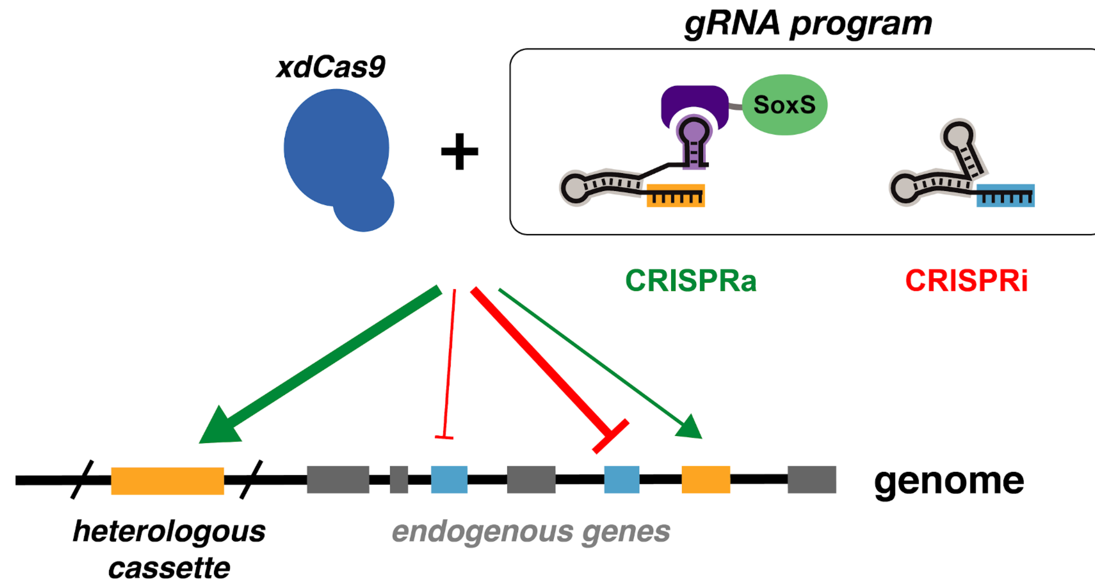
Milestone 2.1.3: CRISPRa from genomic promoters can be achieved with effective targeting rules.

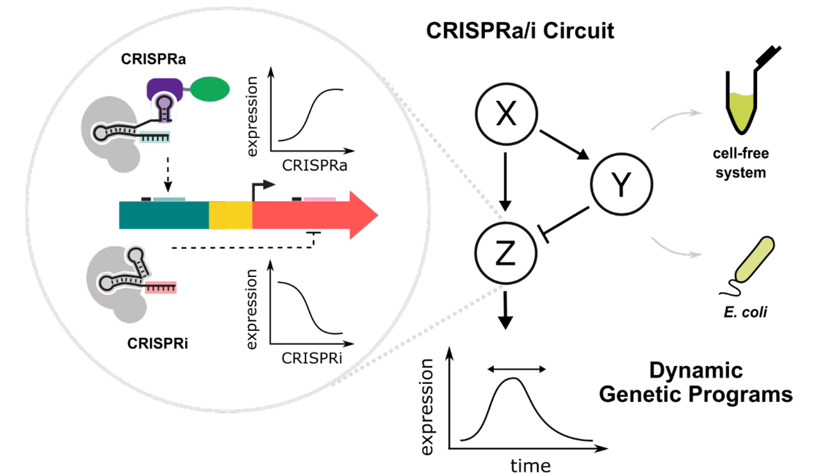
2 - Progress and Outcomes: Task 3. *P. putida* CRISPRa/i tool development

Novel tools for CRISPRa at engineered promoters

Emerging capabilities for CRISPRa at genomic promoters

Integrate CRISPRa with CRISPRi for dynamic expression programs





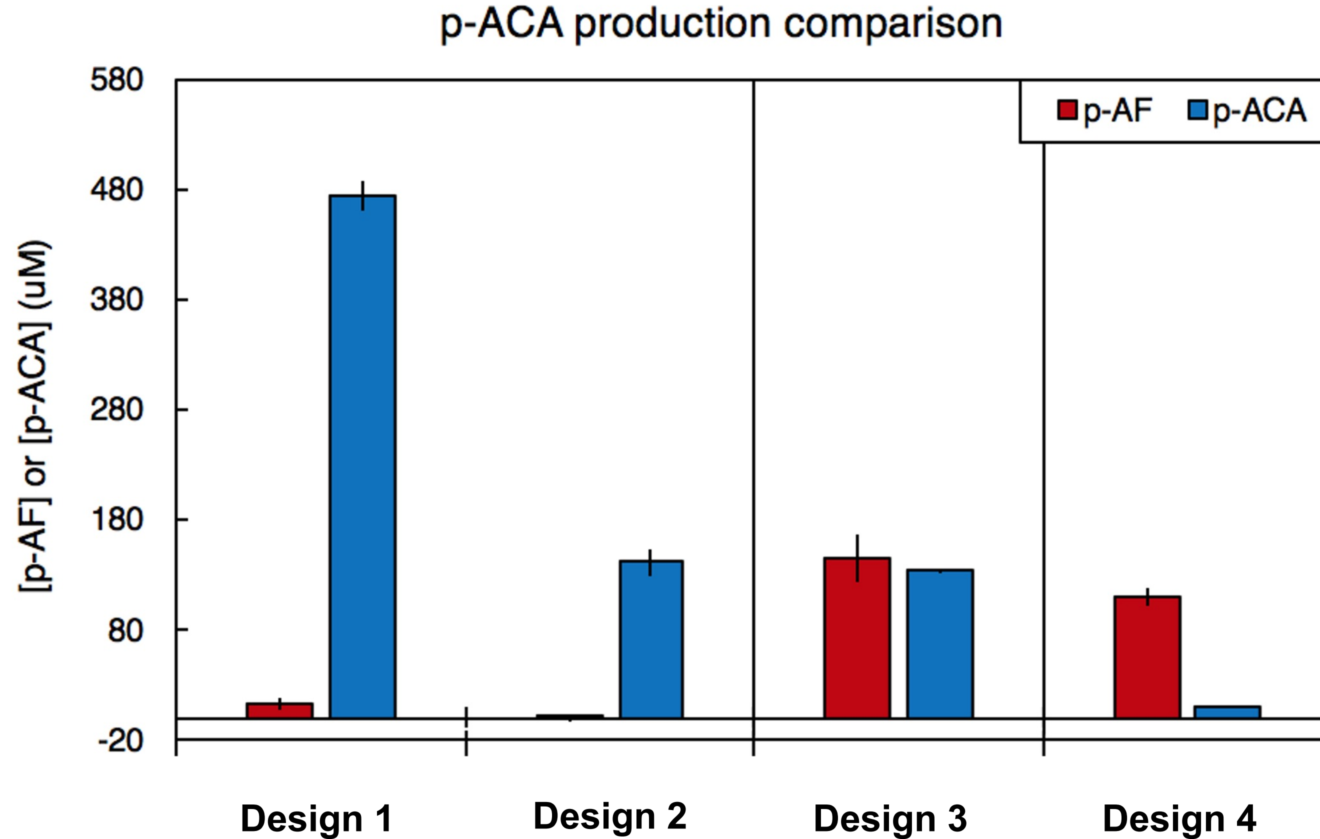
Kiattisawee et al. *Metabolic Engineering* 2021.

Kiattisawee et al. 2022. *ACS Synth. Biol.*

Tickman, Alba Burbano et al. 2022. *Cell Systems.*

Milestone 3.1.1: Multi-gene CRISPRa/i programs can be encoded as genetically-compact sets of guide RNAs.

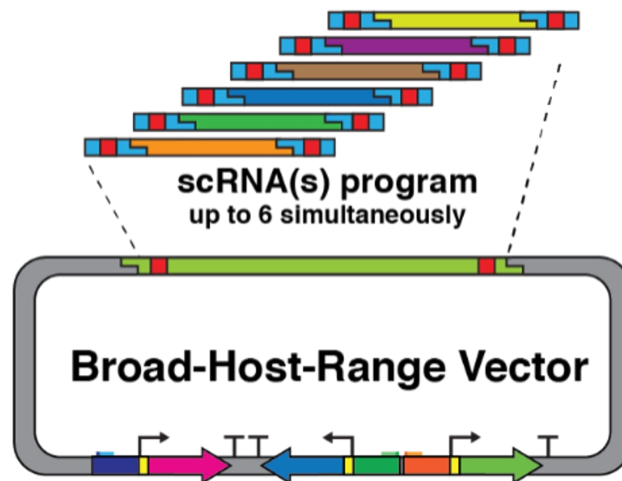
4-ACA production in *P. putida* KT2440 with 3/4-guide program directed CRISPRa of 6/7 genes



Milestone 2.1.2: CRISPRa-directed production of 4-ACA directly from glucose in *P. putida* KT2440.

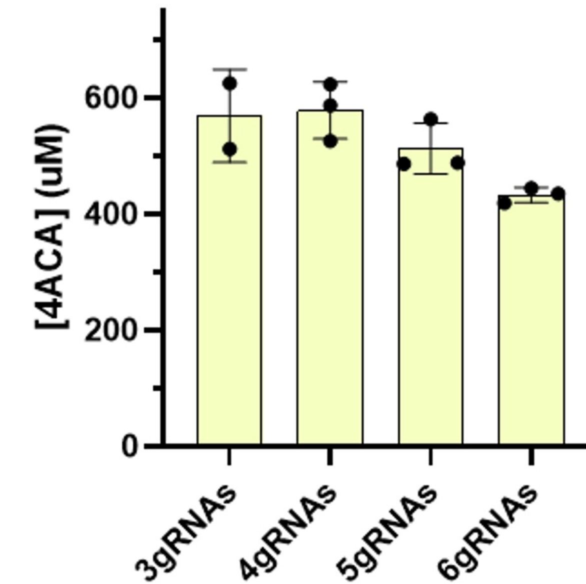
2 - Progress and Outcomes: Tasks 2/4 Multi-gene CRISPRa/i expression programs *P. putida*

5 day build events for creating 6-guide CRISPRa/i expression programs



5 day build event

4-ACA production with multi-guide CRISPRa/i programs



Milestone 3.1.1. Modular genetic constructs for multi-guide expression

Go/No-Go 4.2.3: >30% increase in DBTL cycle efficiency compared to 5 x 1-gene build events in *P. putida* KT2440.

2 - Progress and Outcomes: Task 2. Implement CRISPRa/i perturbations to train ML models

DBTL cycle 0 with single gene perturbations

Genome Scale model → *FVA* → Gene targets

Pathway genes → Gene targets

Gene targets → *CRISPRa/i program engineering* → Engineered *P. putida* strain inputs

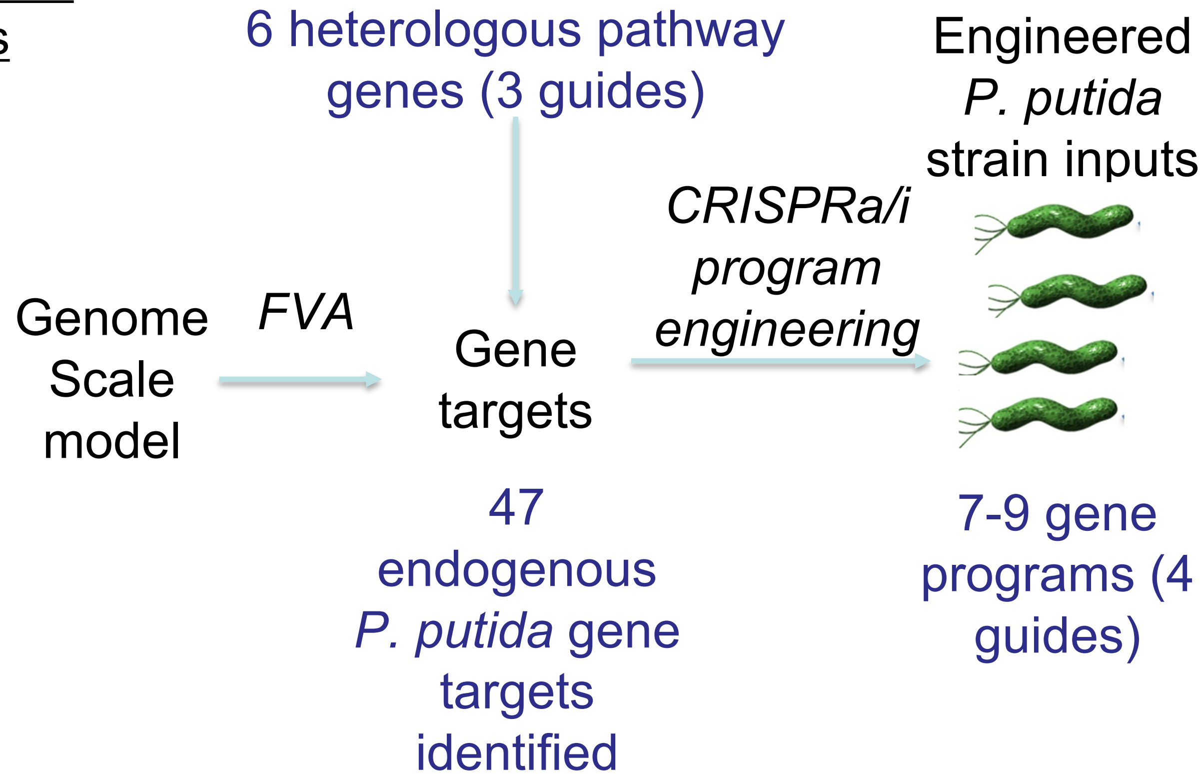
Engineered *P. putida* strain inputs → *Transcriptomics* / *Metabolomic production analysis* → Multi-omic profile outputs

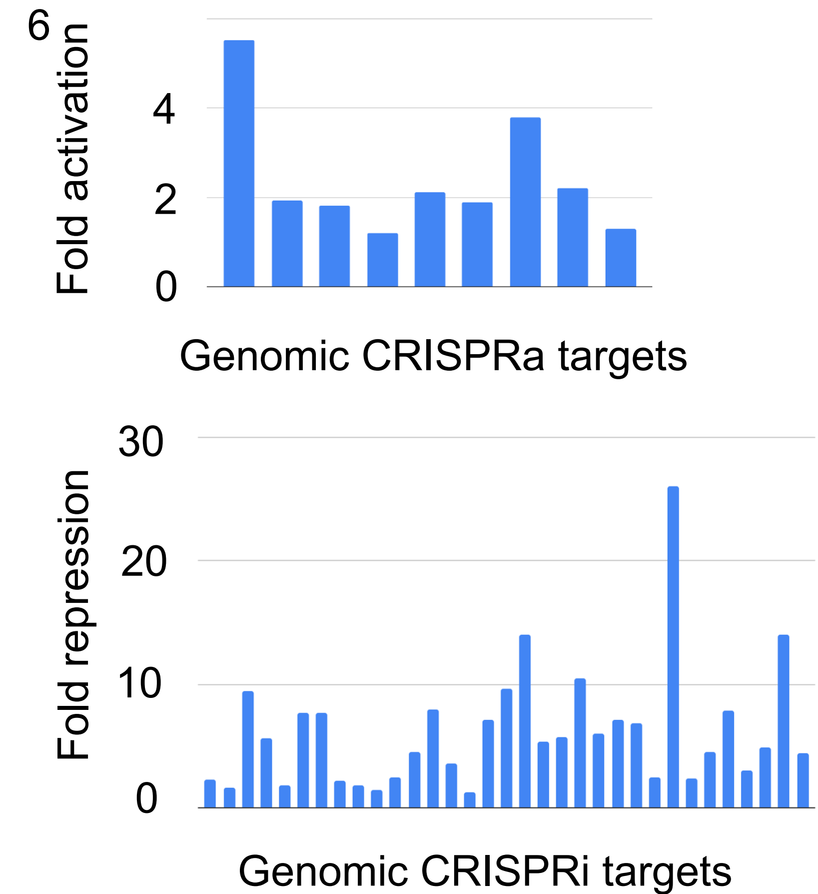
Multi-omic profile outputs → *Machine learning & mechanistic modeling* (art)

Recommendations for next cycle (P1, P2, P3) → *CRISPRa/i program engineering* → Engineered *P. putida* strain inputs

Milestone 2.2.1: Generate CRISPRa/i targets through flux variability analysis.

2 - Progress and Outcomes: Task 2. Implement CRISPRa/i perturbations to train ML models





SMART Milestone 2.2.2: Implement 20 (proposed), 42 (actual) single gene CRISPRa/i perturbations in *P. putida*.

2 - Progress and Outcomes: Task 2. Implement CRISPRa/i perturbations to train ML models

DBTL cycle 0 with single gene perturbations

4-ACA production in *P. putida* KT2440

p-AF
p-ACA

[p-AF] or [p-ACA] (uM)

800
700
600
500
400
300
200
100
0

Subset of 7 to 9 gene programs (4 guides)

Single gene CRISPRa/i perturbations have different impacts on accumulation of 4-AF intermediate and 4-ACA product.

2 - Progress and Outcomes: Most important technical accomplishments achieved

Task 2 *P. putida* CRISPRa/i tool and 4-ACA production strain development

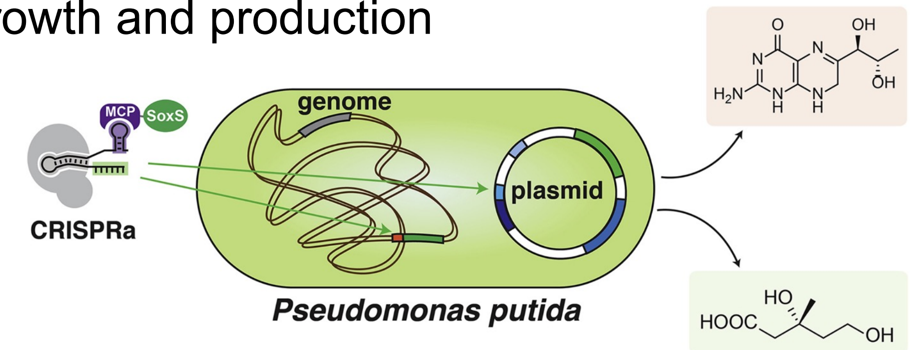
- Developed genetic tools for activating engineered and genomic promoters
- Achieved direct microbial bioproduction of 4-ACA from glucose

Subtask 3.1/Milestone 4.2.3 multi-gene CRISPRa/i program engineering

- Created approach for building genetically-compact, multi-guide programs
- Achieved 5-day build events of 6 guide programs, accelerating DBTL cycles >30%

Task 2/Task 4 Model and data-driven DBTL cycle optimization (DBTL cycle 0)

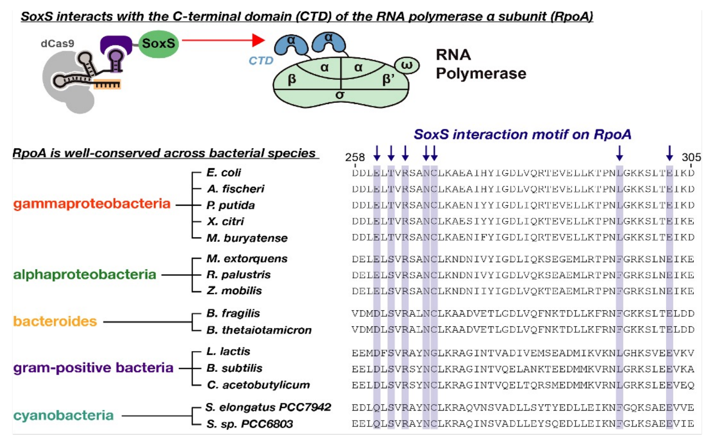
- CRISPRa/i targets selected using genome scale models
- Build 42 strains with multi-gene CRISPRa/i programs perturbing genomic targets
- DBTL cyle 0 CRISPRa/i perturbations generate variations in growth and production



Next: Apply accelerated DBTL cycles to optimized bio-based aromatic production in *P. putida* KT2440.

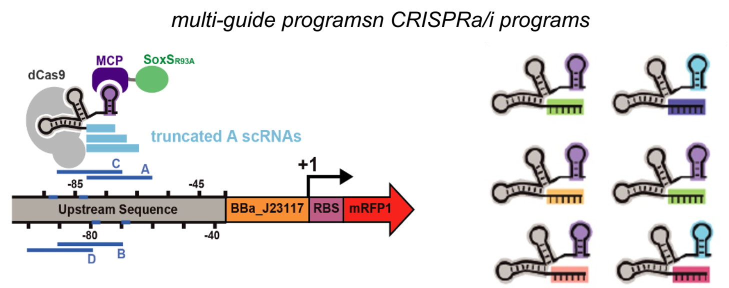
3 – Impact

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

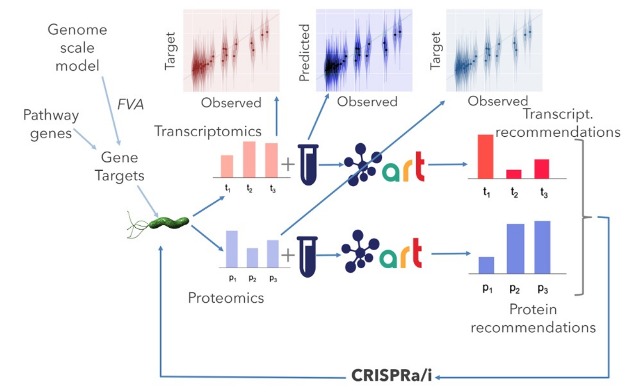


Impact on State of Technology

- 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 ABF hosts



- Genetic technologies to create multi-gene CRISPRa/i programs
  Deliverable: Modular genetic constructs for expressing 6 guide CRISPRa/i programs in *P. putida* KT2440.

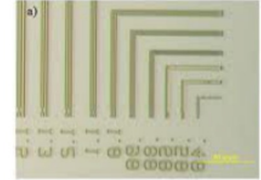


- Improved data- and model-driven microbial engineering
  Deliverable: Workflows for integrating machine learning and kinetic modeling to drive CRISPRa/i program engineering.

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

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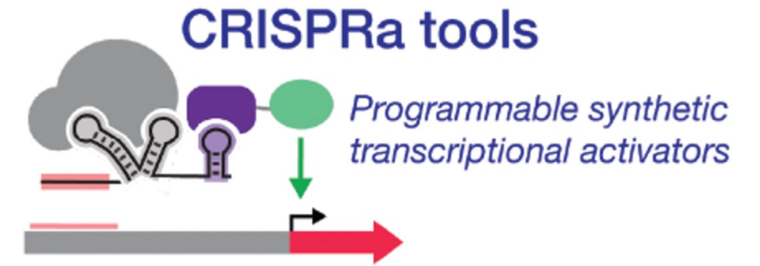
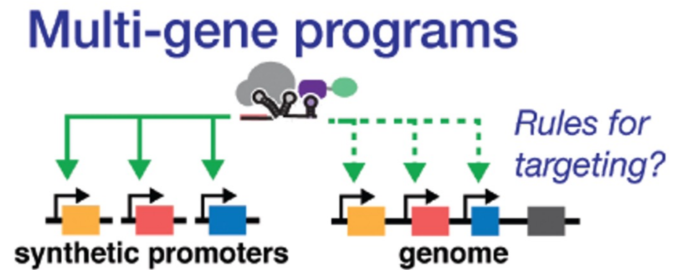
Summary

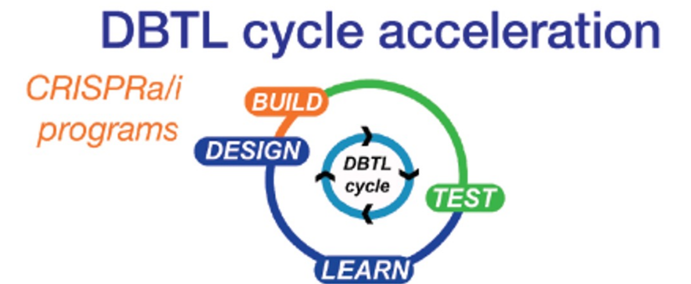
1. Developed and validated a strategy that yields essentially an unlimited supply of orthogonal synthetic promoters for targeted CRISPRa in ABF hosts.



2. Created 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.



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Quad Chart Overview

Timeline
- 1/1/2020
- 12/31/2023

	FY22 Costed	Total Award
DOE Funding	(10/01/2019 – 9/30/2020) *$334K*	(negotiated total federal share) *$1.83M*
Project Cost Share	$144K	$0.46M

Project Partners
- Pacific Northwest National Laboratory
- Lawrence Berkeley National Laboratory

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.

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

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

Additional Slides

Publications, Patents, Presentations, Awards, and Commercialization

Dissemination – Publications and patents

1. 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*. *Metab. Eng.* 2021. 66: 283-295. DOI: https://doi.org/10.1016/j.ymben.2021.04.002. [*24 citations*]
2. Kiattisewee, C.+, Karanjia, A.V.+, Legut, M., Daniloski, Z., Koplik, S.E., Nelson, J., Kleinstiver, B.P., Sanjana, N.E., Carothers, J.M.*, Zalatan, J.G.* Expanding the scope of bacterial CRISPR activation with PAM-flexible dCas9 variants. *ACS Synth. Biol.* 2022. 11, 12, 4103–4112. DOI: https://doi.org/10.1021/acssynbio.2c00405 [*Published November 15, 2022*]
3. Garcia Martin, H.* et al. Perspectives for self-driving labs in synthetic biology. *Curr. Opin. Biotechnol.* 2023. 79, 102881. DOI: https://doi.org/10.1016/j.copbio.2022.102881 [*3 citations*]
4. Shin, J., Carothers, J.M, Sauro, H.* Standards, Dissemination, and Best Practices in Systems Biology. *Curr. Opin. Biotechnol.* [*In press*]
5. Cholpisit Kiattisewee, James M. Carothers, Jesse Zalatan, Ian D. Faulkner, Jason Fontana, Chen Dong. “Genetic manipulation method in bacteria”. U.S. provisional patent application 63/335,143, April 26, 2022. [*Conversion to non-provisional on-going*].

+ = co-first authors
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Dissemination – Presentations by PI Carothers

- Washington University in St. Louis, Dept. of Energy, Environmental & Chemical Engineering, Engineering biology to produce chemicals and materials, 10/22.
- Society for Industrial Microbiology Annual Meeting (San Francisco, CA), Developing multi-gene CRISPRa/i programs to accelerate DBTL cycles in microbes engineered for chemical production, 8/22.
- Cold Spring Harbor Laboratory (Cold Spring Harbor, NY), Engineering biology to produce chemicals and materials, 7/22.
- University of California, Irvine, Dept. of Pharmaceutical Sciences (Irvine, CA), Engineering biology to produce medical-important chemicals and materials, 5/22.
- Northwestern University Center for Synthetic Biology (Virtual), *Developing multi-gene CRISPRa/i programs to engineer metabolism, 11/21*
- mSystems Thinking Session (Virtual), *Developing multi-gene CRISPRa/i programs to engineer bacterial metabolism*, 10/21
- Cold Spring Harbor Asia Conference on Synthetic Biology, Suzhou, China (Virtual), *Developing multi-gene CRISPRa/i programs to engineer metabolism*, 10/21
- 3rd AfroBiotech Conference, Atlanta, GA (Virtual), *Challenges and opportunities with CRISPR activation in bacterial and cell-free pathway engineering,* 10/21
- Build-A-Cell Seminar Series (Virtual), *Multi-Layer CRISPRa/i Circuits for Dynamic Genetic Programs in Cell-Free and Bacterial Systems, 8/21*
- Bioengineering Departmental Seminar, University of Washington (Virtual), *Challenges and opportunities with CRISPR activation in bacterial and cell-free pathway engineering*, 4/21
- Genetics, Cell Biology, Development Seminar, University of Minnesota (Virtual), *Challenges and opportunities with CRISPR activation in bacterial and cell-free pathway engineering,* 3/21
- Synthetic Biology, Cold Spring Harbor Asia, Suzhou, China, *Developing multi-gene CRISPRa/i programs for synthetic cells engineered for bio-production*, 10/2019
- 2nd Annual Synthetic Biology USA Congress, Boston, MA, *Engineering Dynamic Multi-gene CRISPR-Cas Programs*, 5/19

Highlights from Go/No-Go BP2 Intermediate Verification (Summary)

The following observations describe how the project is meeting Topic Area 7b Multi-Topic FOA requirements:

- The project is utilizing *P. putida* and a second ABF host organism (likely *A. baylyi*) as nonmodel host organisms with industrially relevant production advantages over *E. coli* and *S. cerevisiae*.
- The project is utilizing the unique capabilities of the ABF.
- The project will progress to testing production of 4-ACA in bioreactors using biomass intermediates as feedstock.
- The project has met the goal for increased DBTL cycle efficiency in *P. putida*.

BP2 Intermediate verification (virtual) Sept. 22 and Nov. 22, 2022.

Highlights from Go/No-Go BP2 Intermediate Verification (Project recommendations)

1. While this project is behind schedule and has not completed all of their BP2 scope, it has made significant progress towards its goals and the verification team sees value in the continuation of this project. Thus, we are recommending the continuation of this project into the next budget period with some modifications in scope, as described below, to maximize the value of the outcomes for the project team, BETO, ABF and the research community at large.

2. The verification team recommends that the scope of BP3 is shifted to put increased emphasis on maximizing the learnings from the *P. putida* CRISPRa/i platform and associated DBTL cycles, and significantly less resources are devoted to developing CRISPRa/i in a second ABF host. As part of this redefining of BP3 scope, we recommend changes and considerations to the project scope and SOPO. The report contains a full description of recommended changes.

3. The verification team recommends that biomass intermediates (DMR hydrolysate or others) are used as a feedstock for 4-ACA production in subtask 4.2 to fulfill the requirements the FOA. We recognize that production will likely be lower in biomass intermediates than in rich media, and think that a comparison between the different growth media would be useful to inform future studies.

BP2 Intermediate verification (virtual) Sept. 22 and Nov. 22, 2022.

Responses to previous reviewers' comments

We sincerely appreciate the previous reviewers' overall enthusiasm for the project and the constructive feedback that we received about focusing the work to maximize the generalizability of the approaches and the likelihood that the technologies could have industrial impact.

Two of the reviewers raised interesting points about the degree of activation and repression that can be achieved with CRISPRa/i in bacteria.

- "Looking forward, there is mention of a number of "learn" goals, including defining rules to de-risk CRISPRa designs, using ART to survey the combinatorial space, and multi-omic analysis, but no progress was reported in these areas yet. <u>They may be hampered by technical limitations, say, if they require a larger dynamic range than is accessible via the CRISPR tools or if they are easily confounded by off-target regulation</u>." [Emphasis added].

- "Model genetic organisms can routinely alter 3-5 sites at once through CRISPR engineering. This project is one way to reach such a goal, but <u>I see restrictions to the amount of over expression you can achieve through this system, as well as questions of the degree of inhibiting gene expression, as opposed to a true knock out.</u> Again, it's good to see this project as a risk mitigation to achieving multi-locus engineering directly with CRISPR, as long the direct CRISPR work will be resources as part of host onboarding."

Although we are not yet in aposition to present our latest results in public settings (many were presented in the Fall '22 Intermediate Verification), we have seen that generally the amount of CRISPR-directed activation and repression achieved at genomic targets does not appear to be a limitation. In fact, we have seen just the opposite: that CRISPRa driving high levels of expression from some genomic loci can be too strong. In those cases, we have seen that CRISPRa tuned to generate lower levels of target gene expression can be beneficial. By the same token, we have seen that excessive CRISPRi-directed repression of genomic targets can result in aberrant regulatory feedback except when 'weaker' CRISPRi is applied. We also note that compared to true knockouts, partial CRISPRi can be applied to perturb the expression of essential genes. None of this is to say that there will never be limitations in how CRISPRa and i can be applied, but rather that we expect that capabilities for tuning quantitative variations in CRISPRa/i will become increasingly important as the technologies continue to develop.

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