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High Throughput Whole Cell Screening for Optimized Metabolic Flux

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Many Products in a Barrel of Oil

Source: Petroleum Service Company.

<http://www.industrialoutpost.com/oil-barrel-42-gallon-breakdown/>

Replacing the Barrel with Biology

Source: Petroleum Service Company.
<http://www.industrialoutpost.com/oil-barrel-42-gallon-breakdown/>

Better Than the Barrel

Source: Petroleum Service Company.
<http://www.industrialoutpost.com/oil-barrel-42-gallon-breakdown/>

Algae

(Cyano)Bacteria

Petroleum Replacement Products:
Biofuels and Bioproducts

***Food
Feed
Pharma***

The Role of LANL in Biomanufacturing

- LANL's Energy Security mission includes securing America's domestic energy needs
- We are leveraging capabilities developed in more 'traditional' LANL mission space (public health, defense, chemistry, materials) to advance the bioeconomy

Primary Challenge of Bioproducts: Cost

Our overall goal is to develop tools and approaches that demonstrate that biofuels and bioproducts can be made in an industrially relevant way.

“Industrially Relevant” → Show that we can take our laboratory results and translate them to something external stakeholders can use.

3 Overarching Considerations

- 1. Economics:** Show growth and product titers that will permit cost competitiveness with petroleum.
 - *Productivity: Biomass/growth – Biochemical Composition – Bioproducts/Co-products*
- 2. Scale Up:** Show that strains improved in the laboratory have potential to perform at larger scales.
 - *External Partnerships: Academic and Industrial*
- 3. Non-model Organisms:** Typically using potential production organisms.
 - *Single-celled: Eukaryotic or Prokaryotic*

Outline

- Design, Build, Test, Learn
- High Throughput Whole Cell Screening
- Application Examples
 - Making the Tools
 - Towards “Optimizing” Metabolic Flux
- Conclusions

What is Design Build Test Learn?

The DBTL Cycle Will Look Familiar

Come up with an idea to solve a problem or answer a question

Design

Build

Build and combine your components as needed

Test

Conduct the experiment

Learn

Use your results to figure out what to do next

The DBTL Cycle Will Look Familiar

Come up with an idea to solve a problem or answer a question

Combinatorial Libraries Can Get Large Quickly

- Cannot screen each mutant one at a time
 - *Screen as a population and isolate the best*
 - *Must maintain phenotype-genotype link*

Flow Cytometry is a High Throughput Screening Method

Measurements
Functional Assays

Population
Characterization

- Single cell
- 1000s of cells *per second*
- Quantitative
- Multiparameter
- Nondestructive
- Maintains genotype/phenotype

Track

- Productivity of macromolecules/organelles
- Growth and cell morphology
- Productivity of small target molecules

Flow Cytometry is a High Throughput Screening and *Whole Cell Isolation* Method

- Single cell
- High throughput
- Quantitative
- Multiparameter
- Nondestructive
- Maintains genotype/phenotype

Track

- Productivity of macromolecules/organelles
- Growth and cell morphology
- Productivity of small target molecules
- Couple the above with fluorescence activated cell sorting (FACS) to isolate top performing populations

Assays for Tracking Productivity/Health in Algae

Forward Light Scatter	Side Light Scatter	Red Fluorescence	Green Fluorescence	Orange Fluorescence	Green Fluorescence	Green Fluorescence
Size	Granularity	Chlorophyll	Lipids (when stained)	DNA (when stained)	Metabolic Activity (when stained)	pH (when stained)

- Track various macromolecules and organelles, as well as specific cellular activities/function.
- Developing additional assays (e.g. proteins, carbohydrates, reactive oxygen species)
- Can be applied to laboratory cultures as well as to samples taken from outdoor ponds

Claire Sanders, Scott Twary

Improving Algae Lipid Accumulation for Fuels

- Demonstrated in several strains
- Natural genetic variation, random mutagenesis, or libraries of engineered cells
- Depends on having a fluorescent dye that interacts with the product of interest

**But What If We Don't Have a Fluorescent
Dye for Our Target?**

Sorting Can be Conducted on Inherent Properties

Break GMDs, test growth

Or Target Production Can be Linked to a Fluorescent Reporter Using a Biosensor

- Start with naturally occurring transcription factors and their promoters
- Custom built to be **specific** and have good **dynamic range** & **relevant sensitivity**
- Keeps genotype-phenotype without needing to apply a dye

Variations Can be Made as a Combinatorial Library of Enzyme(s)

- Plug in enzyme variants for phenotypic variation
- Increase/decrease enzyme concentration (next slide)
- Sense the product using TF of choice
- FACS for optimized pathway

Variations Can be Made as a Combinatorial Library of Enzyme(s) or Regulatory Elements

Cis-repressors

Application Examples

<https://agilebiofoundry.org/>

Ramesh Jha
Niju Narayanan
Scott Hennelly
Naresh Pandey
Carol Kay Carr

Gregg Beckham
Gayle Bentley
Christopher Johnson

THE Ames Laboratory
Creating Materials & Energy Solutions
U.S. DEPARTMENT OF ENERGY

Adam Guss
Joshua Elmore

Producing Muconate in *Pseudomonas putida*

- Muconate is a precursor to adipic acid and other precursors for bioplastics
- Most recent description using model aromatic compounds (Salvachúa *et al.*, Green Chem 2018)
- Now working glucose from deconstructed biomass

Generated Four Biosensors for *P. putida*

- Muconate is a precursor to adipic acid and other precursors for bioplastics
- Most recent description using model aromatic compounds (Salvachúa *et al.*, Green Chem 2018)
- Now working glucose from deconstructed biomass

“Optimizing” Metabolic Flux = Carbon Partitioning

Addition/
deletion of
genes

CJ200: Yield 5%
(50 mM Glucose)

CJ522: Yield >35%
(30 mM Glucose)

Improve Yield

Improve Growth While Keeping Yield

Improve Yield: Second Shunt of Carbon from Shikimate Pathway – Enzyme is Product Inhibited

PDB code 1JD3
 $K_p = 2 \mu\text{M}$

Severe product inhibition

We Have a Sensitive and Specific 4HB Sensor

- 4HB binds to PobR which binds to a modified promoter (pobR-DM)
- 4HB sensor responds to 4HB but not benzoate

(b) Sensor

Synthetic genetic circuit
in *Pseudomonas putida*
KT2440: 4HB sensing

Positive Feedback Design for Detecting Active UbiC

(c) Sensor + Enzyme

Synthetic genetic circuit in *Pseudomonas putida* KT2440: 4HB conversion and sensing

Positive feedback regulation

- Sensor doesn't respond to benzoate, but UbiC does
- Use benzoate as surrogate inhibitor to UbiC to screen for mutants less sensitive to 4HB

Mutations in UbiC Were Created and Transformed into *P. putida*

- Conservative mutations in the flap region (Han *et al.*, 2016)
- Diversity of ~400
- Add 10mM benzoate to inhibit the different UbiC variants (hopefully to different extents)
- Mutants with relieve product inhibition should make 4HB, not be inhibited, turn on GFP, and make more of that UbiC

GFP is Expressed More Highly in Mutants Than in Wildtype Under Benzoate Inhibiting Conditions

L30A is from Han *et al.*, 2016

K_p for 4HB is Increased Significantly in Mutants

UbiC Variant	Mutations	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1}\text{M}^{-1}$)	K_p (μM)
Wild Type	None	32.9 ± 2.8	0.37 ± 0.04	1.1×10^4	2.4 ± 0.2
UbiC-L30A	L30A	74.1 ± 8.7	0.64 ± 0.07	8.6×10^3	13.2 ± 1.0
UbiC-C11	E31Q/I78V/ L80V	54.7 ± 3.4	0.51 ± 0.04	9.3×10^3	4.7 ± 0.6
UbiC-C21	M34V/I78V	90.2 ± 7.8	0.67 ± 0.06	7.4×10^3	17.8 ± 1.4
UbiC-C22	E31Q/M34V	147.7 ± 9.4	1.04 ± 0.09	7.0×10^3	19.3 ± 2.5

- Able to screen ~400 mutants at once, aim to do more in a second experiment
- Easily able to pull out double mutants
- Identified new mutant and demonstrated a new method for finding mutations that relieve product inhibition *in vivo*.

L30A is from Han *et al.*, 2016

Plasmid Based UbiC Variants Increase Titters and Yields of Muconate

Genome Integrated UbiC Variants Increase Titters and Yields of Muconate

Recovering Growth While Maintaining Muconate

- Knocking out *gcd* (CJ522) led to severely compromised growth
- G. Bentley conducted Adaptive Laboratory Evolution to gain growth
- Transformed muconate sensor into ALE population

Transform Muconate Sensor Plasmid into CJ522 Evolved Population GB052

FACS of GB052 using the Muconate Sensor

Sort top 5%

Sort top 1% and bottom 5%

Apparent enrichment
after first sort

After Sorting, Colonies Were Picked for Characterization

Bottom 5% Colonies = “D”, “E”

Large Colonies = “A”
Small Colonies = “B”

Confirm in shake flask for muconate production in M9 media + 30 mM Glucose
(Quantitatively using HPLC)

Subset of LANL Growth and Muconate by Biosensor Detection (GFP Fluorescence)

Muconate production by HPLC

Muconate yield similar to parent population GB052 and CJ522
10 mM Muconate = 33% yield
LANL Sent 8 clones to NREL for further study

Growth of Cell Sorted Clones at NREL More Varied but Cluster into “A,D,E” and “B”

Max Muconate of Sorted Clones May be Higher than GB045 in Mid-Stationary Phase

Genome Sequencing of Cell Sorted Clones from GB052 Population

Strains Sequenced

- **CJ522** (in July 2017)
- **GB52-A1**
- **GB52-A8**
- **GB52-B9**
- **GB52-B10**
- **GB52-B11**
- **GB52-D10**
- **GB52-E2**
- **GB52-E3**

Cluster 1: A-, D-, E-series

- **Edd operon promoter mutation**
 - C to T mutation
 - Generates additional σ_{70} promoter

Cluster 2: B-series

- **~36 kb chunk deleted**
- **Multiple key genes deleted**
 - zwf-2 (glucose 6-phosphate dehydrogenase 2)
 - spp (sugar phosphate phosphorylase)
 - Majority of glycogen biosynthesis gene cluster
 - gnd (6-phosphogluconate dehydrogenase)

Δgnd in CJ522 Gives Similar Phenotype to $\Delta hexR$ in CJ522 $\rightarrow$ Sugar Regulatory Genes

Growth Analysis

(M9 + 30 mM glucose – plate reader)

- GB052-B strain growth rate increase is likely due to *gnd* deletion
- Deletion of *gnd* forces carbon through ED pathway, increasing KDPG (2-keto-3-deoxy-6-phosphogluconate) and therefore likely reducing HexR repression

Δgnd in CJ522 (JE3943) Gives Similar Phenotype to $\Delta hexR$ in CJ522 (GB062) $\rightarrow$ Sugar Regulatory Genes

Growth

Muconate Production

(M9 + 30 mM glucose - shake flask)

- Collaboratively succeeded in generating new strains with increased growth while keeping high muconate titers
- Results converged on glucose metabolism regulation being a bottleneck for growth
- Conducting fermentation experiments next (NREL) to get productivity values

Learnings from 1st Time Application of Muconate Sensor

Bottom 5% Colonies = “D”, “E” **Large Colonies = “A”, Small Colonies = “B”**

- A, D, and E strains turned out to be **genetically identical**, and we only saw two genotypes overall, indicating little genetic diversity across the histogram/population (at least after the first sort)
- Supported by the relatively little phenotypic variation in the sorted clones compared to GB052 (parent) or GB045 (best isolated at NREL)
- Resulted in the generation of a new, *less sensitive variant* of the muconate sensor, to better distinguish among a population of all relatively high performers

Alleviate Growth Defect Using Riboregulator Knockdowns

Knockdowns
instead of
knockouts

Autoinduction- Muconate feedback pathway induction using T7 RNAP and muconate sensor

- All exogenous genes are under T7 promoter control
- T7RNAP is only turned on when sufficient muconate production occurs (due to promoter leakiness)
- Ideally this permits improved growth early on, and then when cells build up some muconate, the muconate pathway full turns on and shunts C away from growth.

System is Active But Producing Muconate at ~50% of Expected

(+) (1) (2) (-)
T7-CatM T7-CatM NP003.1 KT2440
(Induced)

0 hr

T7-CatM T7-CatM NP003.1 KT2440
(Induced)

12 hr

T7-CatM T7-CatM NP003.1 KT2440
(Induced)

24 hr

48 hr

72 hr

Muconate similar to previous run (<1mM). Still catechol peak is present and pellet is full of black stuff

Conclusions and Future Directions

- Working towards making renewable fuels and chemicals, hopefully one day with added advantages from biology
 - Test is a bottleneck in DBTL
 - Aim to alleviate with flow cytometry
 - Flow cytometry requires a fluorescence or light scatter signal to sort
 - Generate with custom biosensors
 - Applying these approaches to balancing carbon between growth and muconate and increasing yields in new ways.
-
- Continue to develop and demonstrate examples of how biosensors, riboregulators, and FACS can be applied to biomanufacturing
 - Open to Collaboration Discussions

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Energy Efficiency &
Renewable Energy

BIOENERGY TECHNOLOGIES OFFICE

Building the CatM Muconate Sensor

A Highly Sensitive Muconate Sensor

Gene diversification

CJ072 $\Delta pcaHG$ (no *Ptac:catA* ; no $\Delta catRBC$)

CJ184 $\Delta catRBC::Ptac:catA$ $\Delta pcaHG::Ptac:aroY::ecdB::ecdD$

Current detection range <1mM
Ideal detection range needed in ABF >10 mM

Structure based design for optimized sensor

4 mutations in the binding pocket

Library 300

4 mutations in the binding pocket

GB052 "B" Strain Mutations

- zwf-2 (G6P dehydrogenase 2)
- spp (sugar phosphate phosphorylase)
Spp dephosphorylates sugar phosphates when they accumulate to high levels and can cause a futile cycle → avoid by gene deletion.
- Majority of glycogen biosynthesis gene cluster
Build up of G6P may promote glycogen and/or trehalose formation which may pull carbon away from ED pathway waste energy
→ deletion of pathway would reduce this effect
- gnd (6-phosphogluconate dehydrogenase)
Deletion of gnd may prevent additional accumulation of PPP intermediates that are already likely out of balance due to changes made to metabolism for muconate production.

Growth for Autoinduction Strains