Agile BioFoundry

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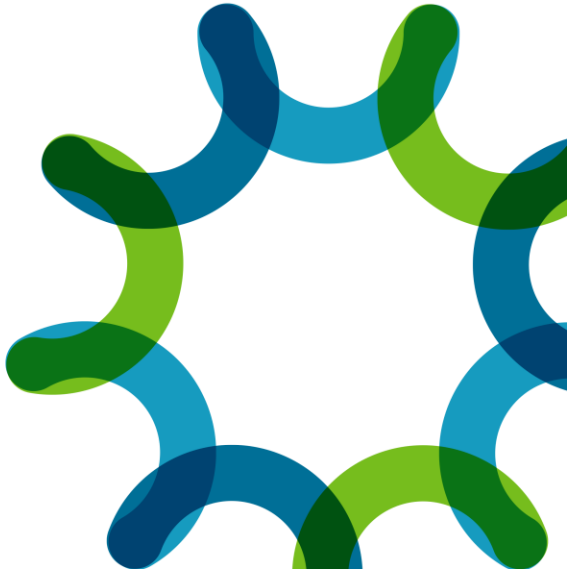
# ABF Demonstration Host: *Aspergillus*

**Jon Magnuson**

- **DBTL: *Aspergillus* Host Lead**
- **DBTL: TEST Lead**

**jon.magnuson@pnnl.gov**

**October 04, 2019**



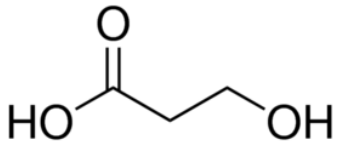
# *Aspergillus pseudoterreus* Introduction

- **Fungi** (*Saccharomyces, Aspergillus, Pichia, Trichoderma*): **industrial workhorses** used for making $ from commodity fuels, chemicals, and enzymes in large bioreactors … e.g., *A. niger producing* citric acid in ≥100,000L airlift reactors = ~3 million ton market

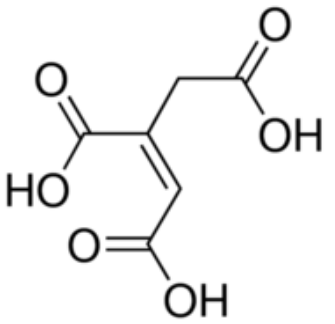
- ***A. pseudoterreus & A. niger****:* genetic tools, genomes sequenced, genome scale metabolic model
- **High flux** from sugars toward beachhead molecules in glycolysis and the TCA cycle, **organic acids**, e.g., *A. pseudoterreus* ATCC 32359 makes 50 g/L itaconic acid
- Grows and produces organic acids at pH 1-3, **free acids, not salts**
  - **Separations**: high titer, free acid, crystallization possible
  - No lime or sulfuric acid input = no waste gypsum
- **Purposes**:
  - Develop **advanced DBTL tools** broadly applicable to *Aspergillus* spp.
  - Show the strength of the platform for producing beachhead molecules (pyruvate, oxaloacetate, AcCoA) leading to **organic acids**

# Demonstration Targets: Organic Acids

- **Target 1** (began FY17): **3-hydroxypropionic acid**
  - Intermediate to acrylic acid and acrylonitrile
  - **Heterologous pathway** (prokaryotic)
  - **Beachheads:** pyruvate, oxaloacetate
  - Nat'l. Labs have a portfolio of IP around acrylonitrile that would benefit from renewable 3HP



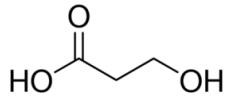

- **Target 2** (began FY18): **aconitic acid**
  - A 6-carbon tricarboxylic acid, like citric acid.
  - Beverage acidulant, industrial chelator/modifier (cement) etc.
  - Central metabolite with **transport limitations**
  - **Beachheads:** pyruvate, oxaloacetate, acetyl-CoA

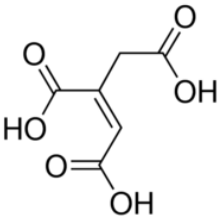



- **Purpose**: industrially relevant organic acid demonstration targets to advance DBTL capabilities for *Aspergillus* and develop bioprocesses

# DBTL Cycles

- **Target 1**: **3-hydroxypropionic acid**
  - *A. pseudoterreus*. Cycle 1-1- through 1-7-
  - *A. niger* “transfer host”. Cycle 9-
- **Target 2**: **aconitic acid**
  - *A. pseudoterreus*. Cycle 2-1- through 2-5-
- **Tool Development**: Cycle 1-8- through 1-11-




Tools

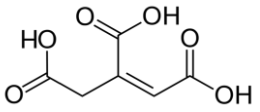
| Cycle | Cycle Description | Host | Target |
|---|---|---|---|
| 1-1-Y_0 | Establish beta-alanine/L-aspartate 3HP pathway | Aspergillus pseudoterreus | 3HP |
| 1-2-Y_0 | Establish beta-alanine 3HP pathway | Aspergillus pseudoterreus | 3HP |
| 1-3-Y_0 | Establish malonyl-CoA 3HP pathway | Aspergillus pseudoterreus | 3HP |
| 1-4-Y_0 | Identify genes involved in 3HP degredation | Aspergillus pseudoterreus | 3HP |
| 1-5-Y_0 | Improve productivy via higher expression of beta-alanine 3HP pathway genes | Aspergillus pseudoterreus | 3HP |
| 1-6-Y_0 | Improve 3HP precursor flux by overexpression of AAT/PYC | Aspergillus pseudoterreus | 3HP |
| 1-7-Y_0 | Improve 3HP production using targets from ANN based learn | Aspergillus pseudoterreus | 3HP |
| 1-8-O_M1 | Improve aspergillus tools (promoters) | Aspergillus spp. | - |
| 1-9-O_M1 | Improve aspergillus tools (splicing motifs) | Aspergillus pseudoterreus | - |
| 1-10-O_M1 | Improve fungal tools (transferable promoters) | Ascomycota & Basidiomycota | - |
| 1-11-O_M1 | Improve fungal tools (synthetic promoters) | Ascomycota & Basidiomycota | - |
| 2-1-Y_0 | Establish aconitic acid production via cad deletion | Aspergillus pseudoterreus | cis-aconitic acid |
| 2-2-Y_0 | Improve flux toward aconitate (rational targets) | Aspergillus pseudoterreus | cis-aconitic acid |
| 2-3-Y_0 | Improve flux toward aconitate (complex design set) | Aspergillus pseudoterreus | cis-aconitic acid |
| 2-4-R_0 | Identify aconitic acid plasma membrane transporter | Aspergillus pseudoterreus | cis-aconitic acid |
| 2-5-R_0 | Overexpress aconitic acid plasma membrane transporter | Aspergillus pseudoterreus | cis-aconitic acid |
| 9-1-Y_0 | Establish beta-alanine 3HP pathway | Aspergillus niger | 3HP |
| 9-2-Y_0 | Improve 3HP precursor flux by overexpression of AAT/PYC | Aspergillus niger | 3HP |

# *Aspergillus pseudoterreus*
# Aconitic acid Target

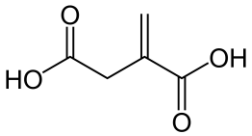
# 2-3-Y_0, Rational designs to improve production

Initial production strain: ***cad***, i.e., deletion of the *cis*-aconitate decarboxylase gene

aconitic acid

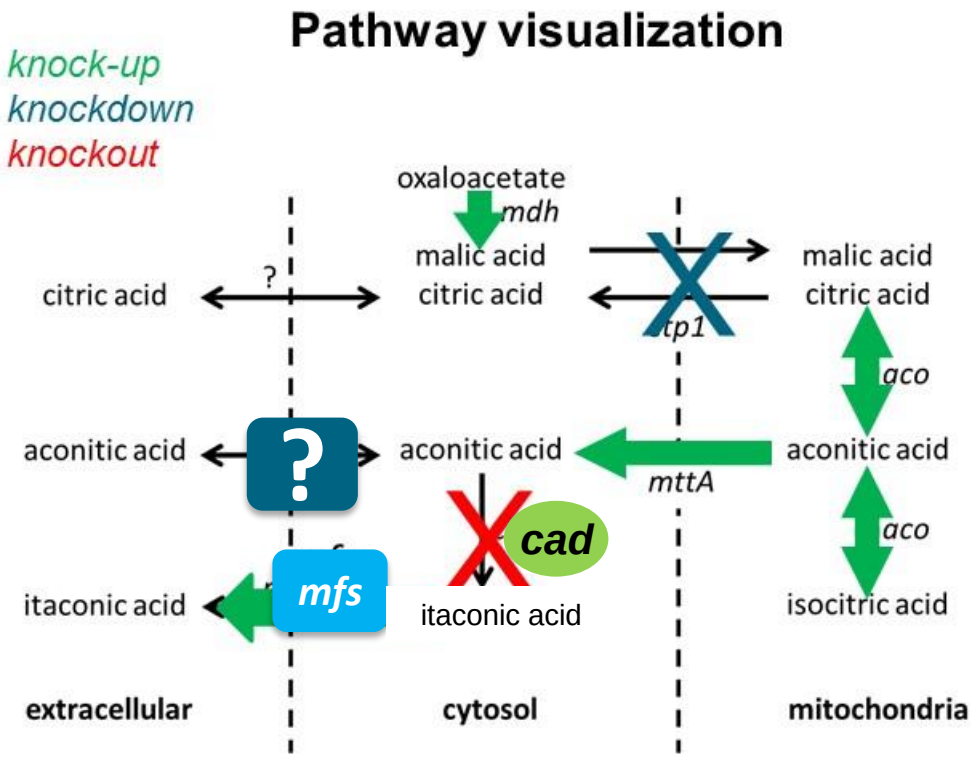


(10g/L)



itaconic acid

(50g/L)

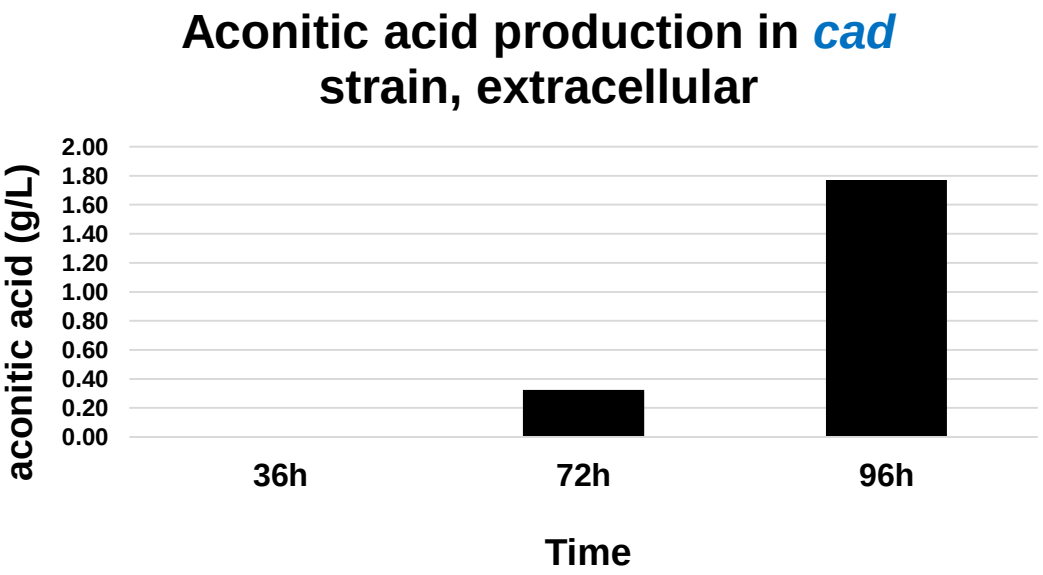


# 2-1-Y_0, Test Learn: multi-omics experiments

**Strains**: WT (itaconic acid) and *cad* (aconitic acid)

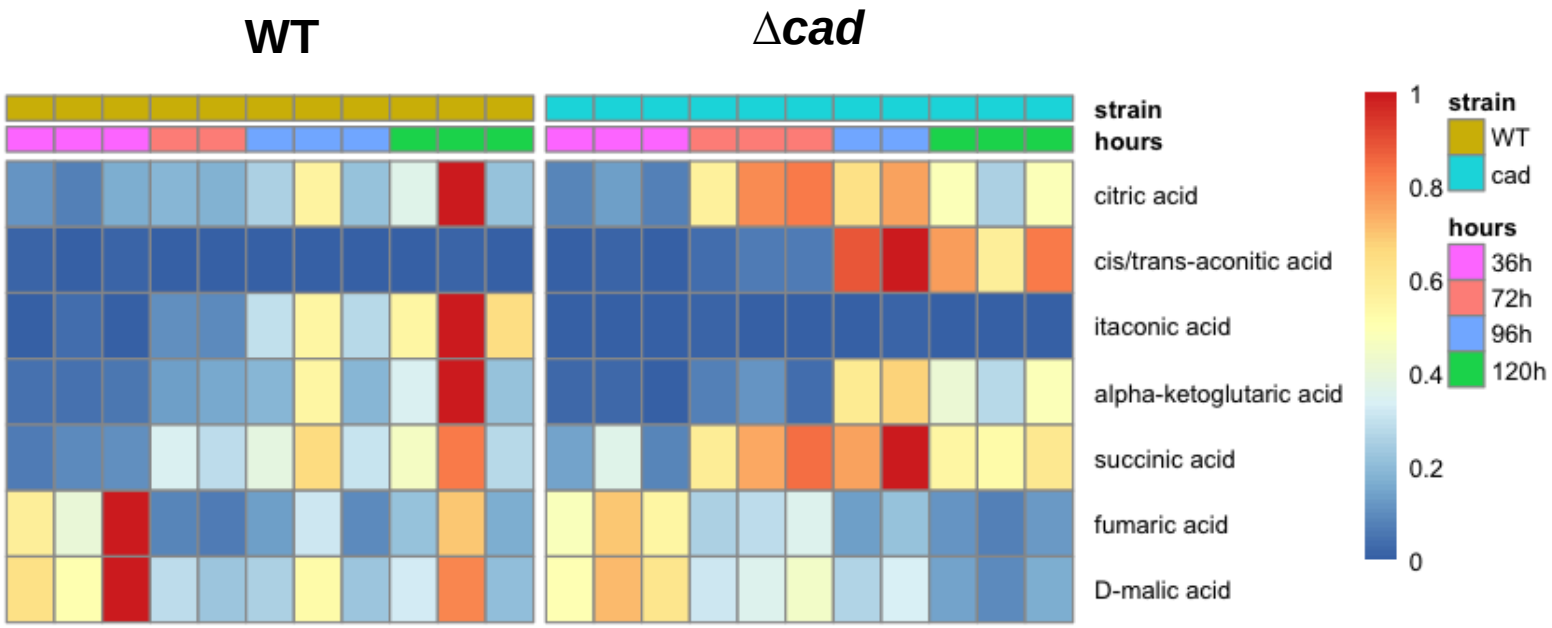
**Test**: transcriptomics, proteomics and metabolomics

**Time points**: 36, 72, and 96 hours



# Intracellular metabolomics

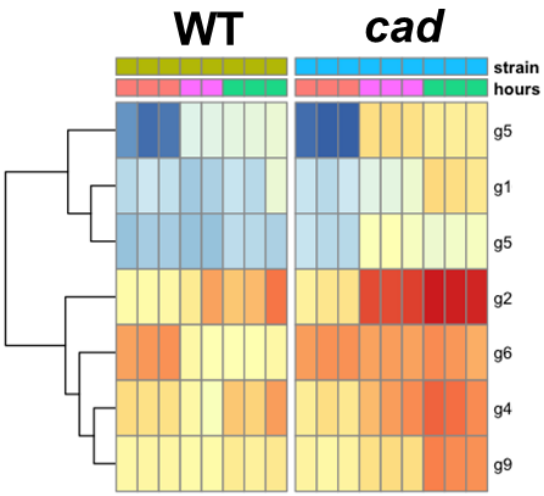
- **Accumulation of itaconic acid in WT strain**
- **Accumulation of aconitic acid and citric acid in *cad* strain**
- **Same pattern observed extracellularly (previous slide)**

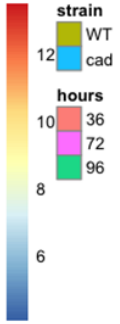


# Transcriptomics

- **MFS transporters upregulated in *cad* vs. WT at 72 and 96 hours**
- **72 = early production phase, 96 = high production phase**




| | Annotation | TCDB |
|---|---|---|
| g5 | Predicted transporter (major facilitator superfamily) | MFS |
| g10 | Monocarboxylate transporter | MFS |
| g5 | Predicted transporter (major facilitator superfamily) | MFS |
| g2 | Predicted transporter (major facilitator superfamily) | MFS |
| g6 | Predicted transporter (major facilitator superfamily) | MFS |
| g4 | Monocarboxylate transporter | MFS |
| g9 | Predicted transporter (major facilitator superfamily) | MFS |

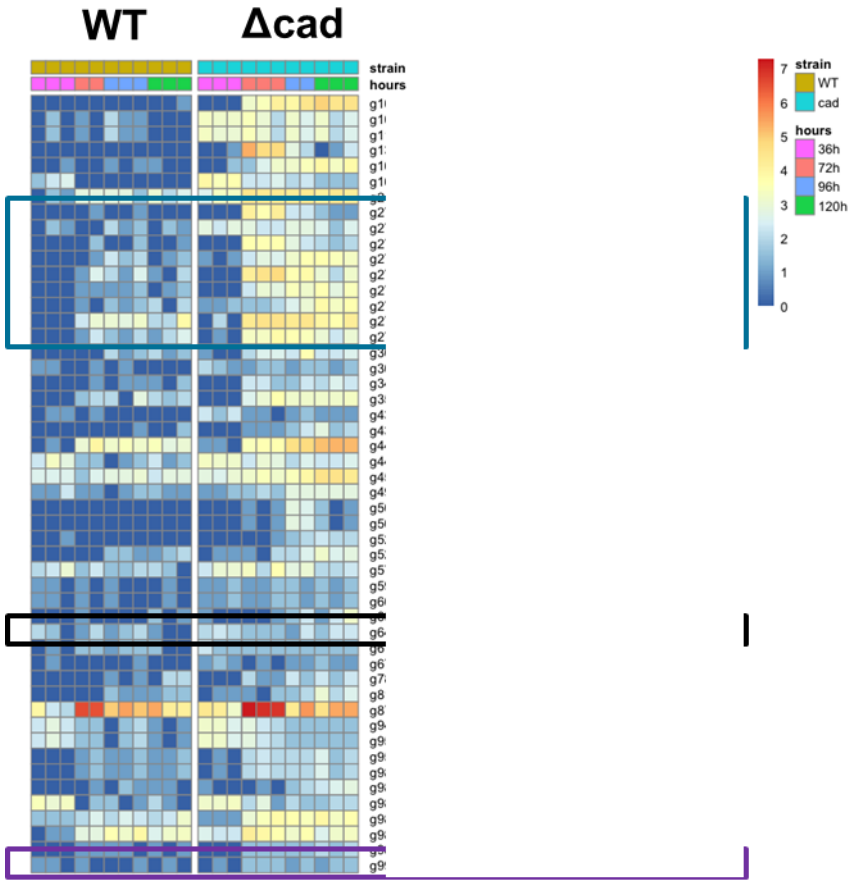
- G2### deletion did not decrease aconitic acid production
- G1###, g4###, and g9### look promising

- *log2 Fold Change > 1 and adjusted p-value < 0.05*

G2### and g6### were also detected in proteomics (data not shown)

# Genes upregulated in *cad*, proteomics



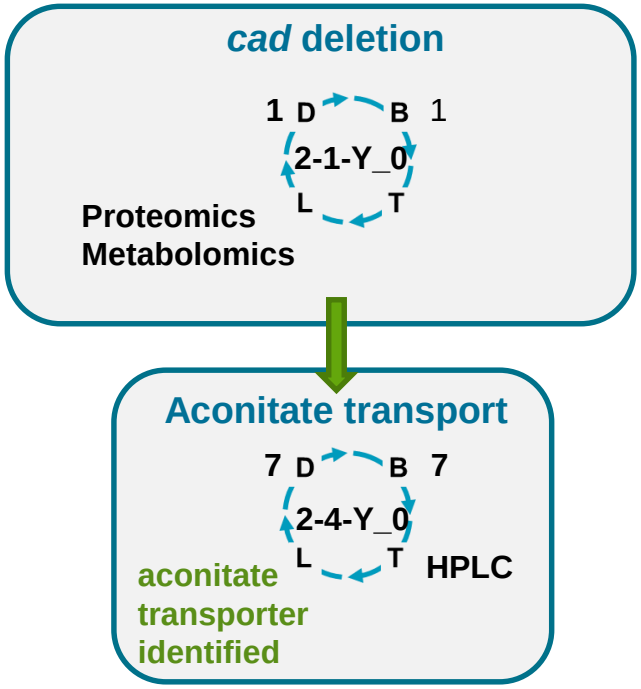

- Inhibitor catabolic gene: over-expression candidate
- Aconitate metabolizing gene: deletion target

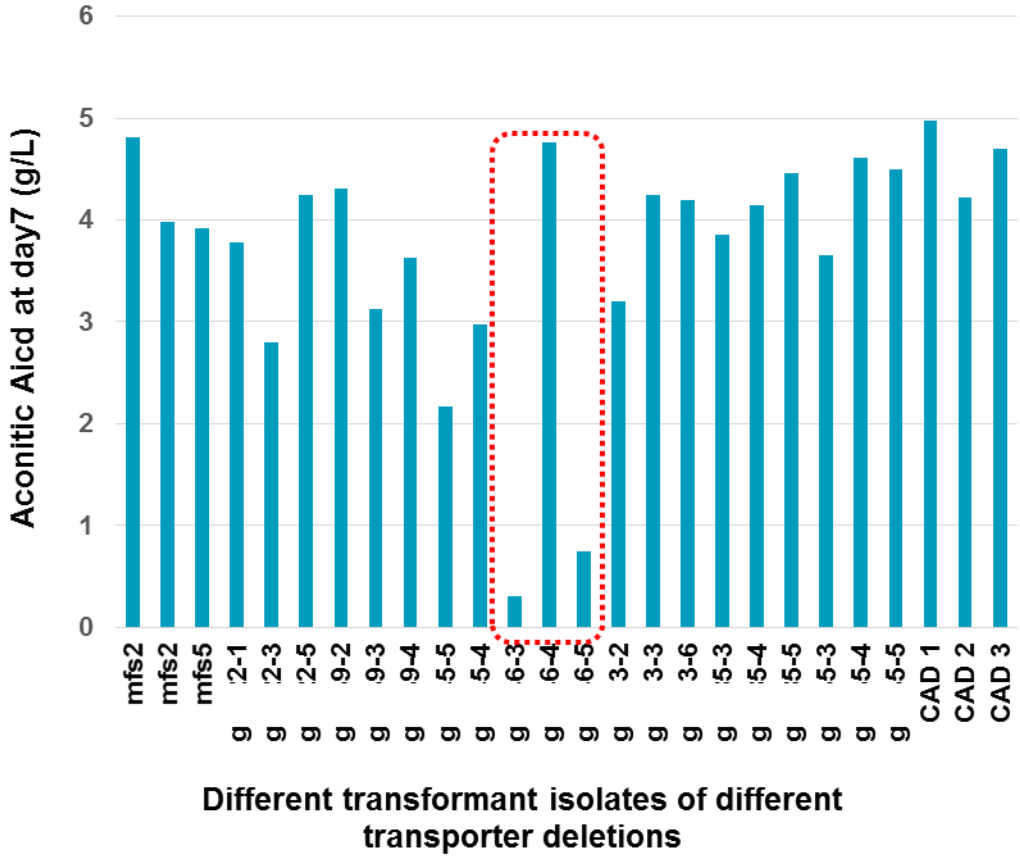
# 2-4-R_0, DBTL:

## Test and Learn Capabilities to ID Transporter Candidates

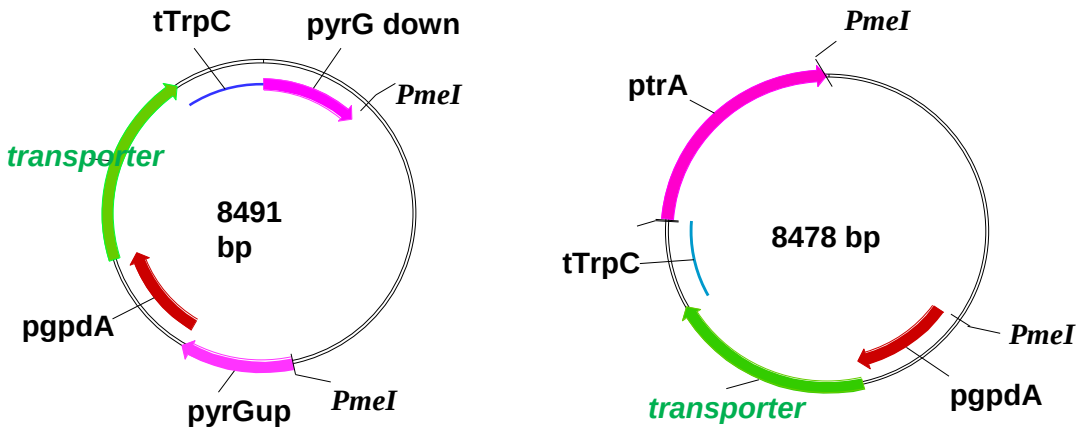
## Design & Build to Confirm




**Potential Transporters Deletion Analysis**



# 2-5-R_0, Design/Build: transporter overexpression



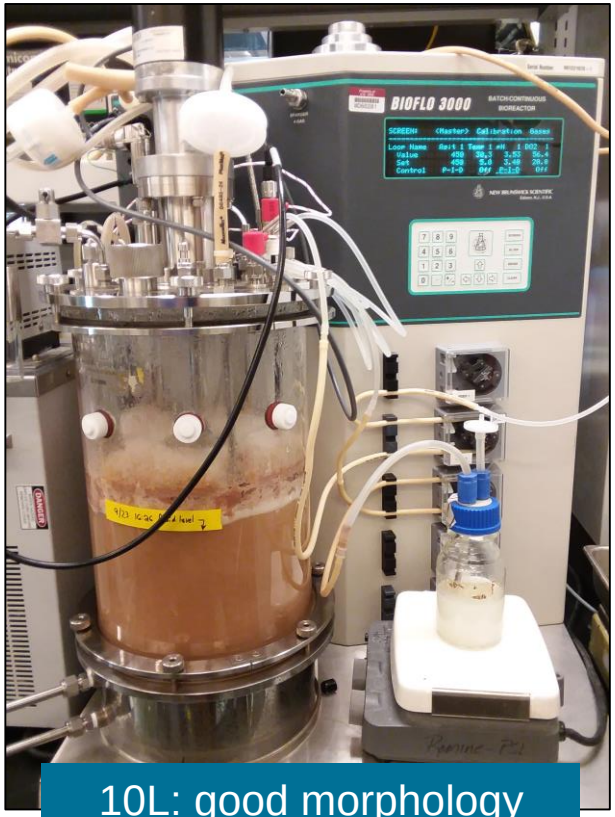

**Aconitate transport**

1 D B
2-5-Y_0
L T

**In progress**

# 2-1-Y_0, Test: *Aspergillus* in small bioreactors

- Scaling down has the advantage that many bioreactors can be run & conditions examined in parallel
- However, Filamentous fungi often exhibit poor morphology in small scale bioreactors (0.5L)




10L: good morphology

Sixfors, 500mL glass: poor morphology

Test *A. pseudoterreus* in the ambr250 with ABPDU
Looking for morphology seen in larger bioreactors

***Process Integration and Scale-Up**: Round Robin tests across the lab partner facilities to identify better reactor configurations, examine reproducibility*

# Future Plans

- **Deletion analysis and over-expression of aconitic transporter candidate genes *and test effect on* aconitic acid production**
- **Deletion or over-expression of candidate metabolic genes from modeling of multiomics data**
- **Test *A. pseudoterreus* in the ambr250 with ABPDU**
- **FY20: Develop *in vitro* CRISPR technique that can delete multiple genes at same time and can be applied to different *Aspergillus* spp.**

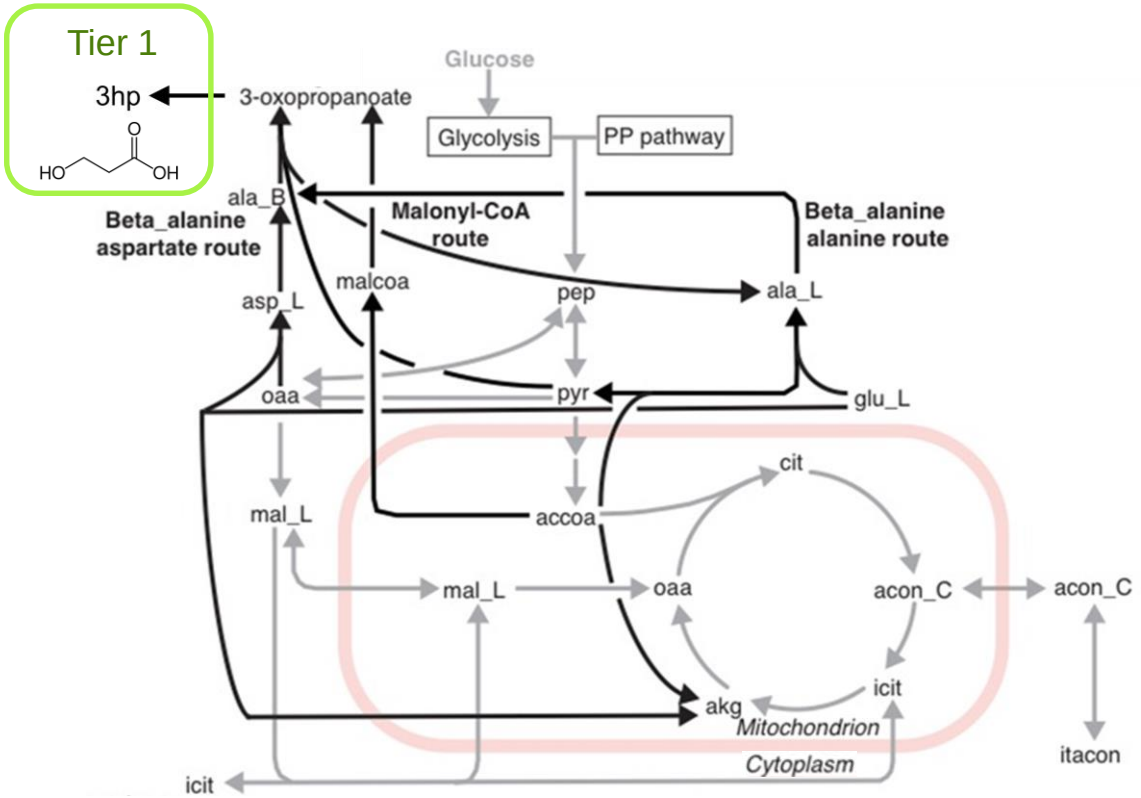
 

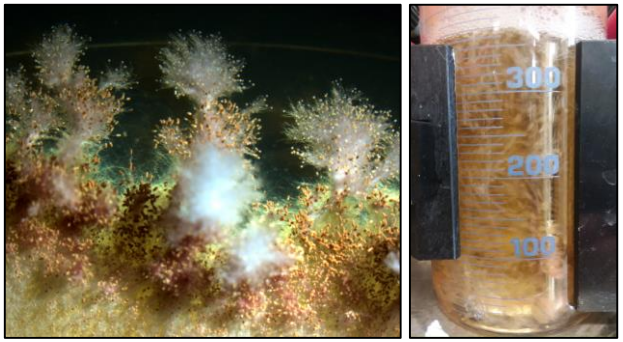
# *Aspergillus spp.*
# 3HP Target

# *Aspergillus sp.* for 3HP production

- β-hydroxy carboxylic acid (pKa 4.5)
- Biorenewable acrylate and acrylonitrile
- biodegradable polymers, poly(3HP)
- Heterologous pathways (prokaryotic)
- Beachheads: pyruvate, oxaloacetate
- National labs have a portfolio of IP around acrylonitrile that would benefit from renewable 3HP

3HP metabolic engineering



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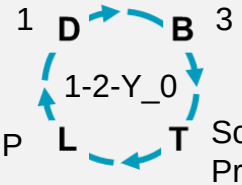
# 3HP DBTL cycles in progress

Establish 3HP production

## β-alanine pathway

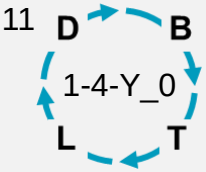



1.5 g/L 3HP
FBA, ANN

Scale-up
Proteomics
Metabolomics

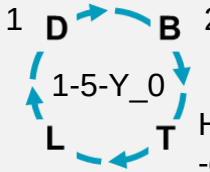
Improve 3HP TRY

## 3HP catabolism




In progress

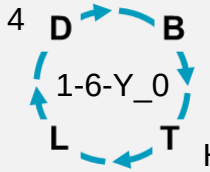
## Pathway expression




3.2 g/L 3HP

HPLC
-omics
in progress

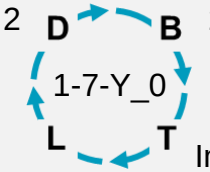
## AAT/PYC overexpression




HPLC

no 3HP
improvement

## ANN targets

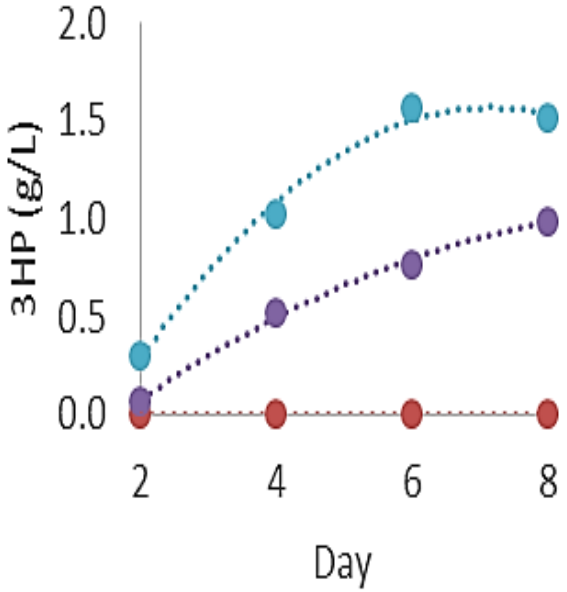



In progress

# Increased Copy Number Correlates with Increased 3HP Titer

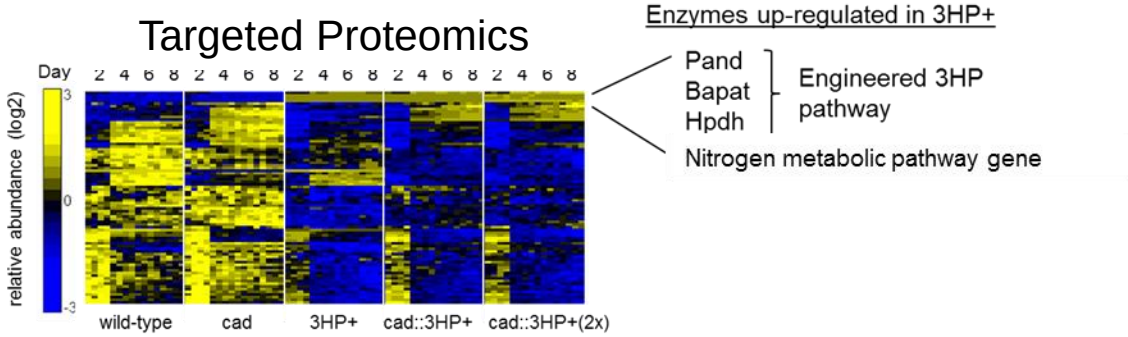
### 3HP productivity

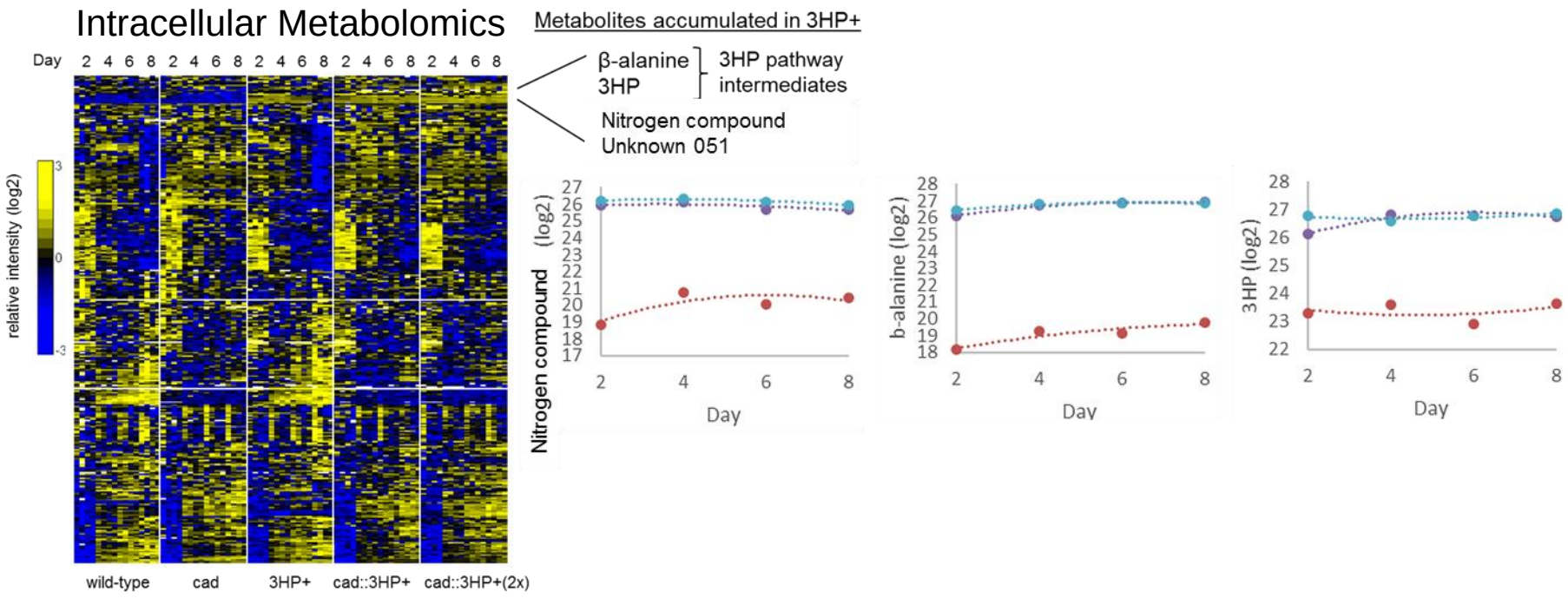

1 copy 2 copies



# 1-2-Y_0, Learn: multi-omics analysis



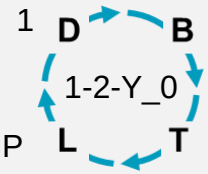



# 1-4-Y_0, Design, Build: Using DIVA at PNNL

Establish 3HP production

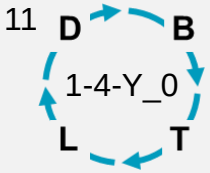
## β-alanine pathway




1.5 g/L 3HP
FBA, ANN
3HP is degraded

Scale-up
Proteomics
Metabolomics

## 3HP catabolism knockouts




In progress

**Projects:** PNNL Build Team +

| Status changed | New status | Updated by | Comment |
|---|---|---|---|
| 7 days ago | Clonal isolation | Kyle Pomraning | |
| 07/15/19 | Assembling parts | Kyle Pomraning | |
| 06/27/19 | PCRing parts | Kyle Pomraning | |
| 06/21/19 | Waiting for reagents | Kyle Pomraning | ordered primers |
| 06/21/19 | In progress | Kyle Pomraning | Construction started. |
| 06/21/19 | Construction requested | Kyle Pomraning | Use charge code: N83885. |
| 06/21/19 | PI approval requested | Kyle Pomraning | Trying out DIVA for PNNL build |

J5 File Load Edit Help

| plasmid backbone | 5' flank | resistance marker | 3' flank |
|---|---|---|---|
| pRF_HU2_BB | ABF_006652 | ABF_006648 | ABF_006663 |
| pRF_HU2_BB | ABF_006653 | ABF_006648 | ABF_006664 |



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# 1-4-Y_0, Design, Build: Using DIVA at PNNL

Establish 3HP production

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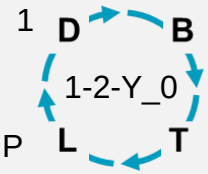


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FBA, ANN
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Scale-up
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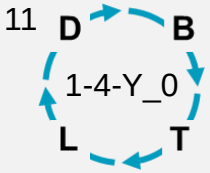


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# 3HP DBTL cycles in progress

Establish 3HP production

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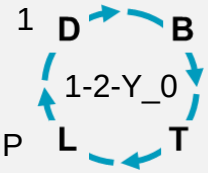
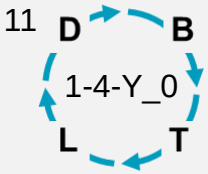



1.5 g/L 3HP
FBA, ANN
3HP is degraded

Scale-up
Proteomics
Metabolomics

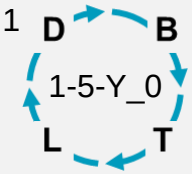
Improve 3HP TRY

## 3OP/3HP dehydrogenase




In progress

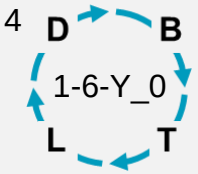
## Pathway expression




HPLC
-omics
in progress

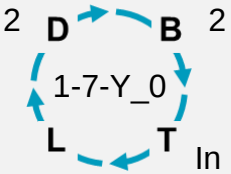
3.2 g/L 3HP

## AAT/PYC overexpression




HPLC

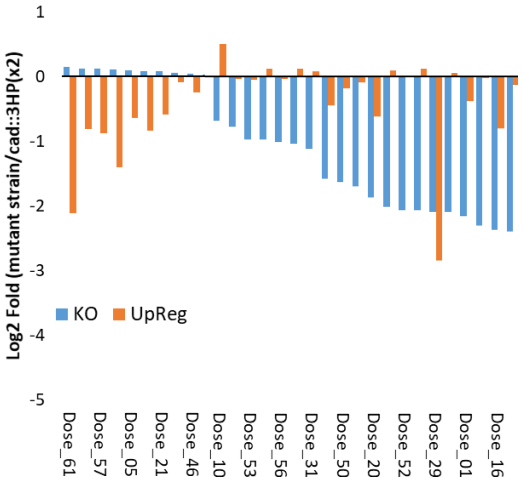
## ANN targets




In progress

# 1-7-Y_0, LEARN: ANN non-intuitive targets

## Predict the effects of genetic perturbation on a trained Artificial Neural Network

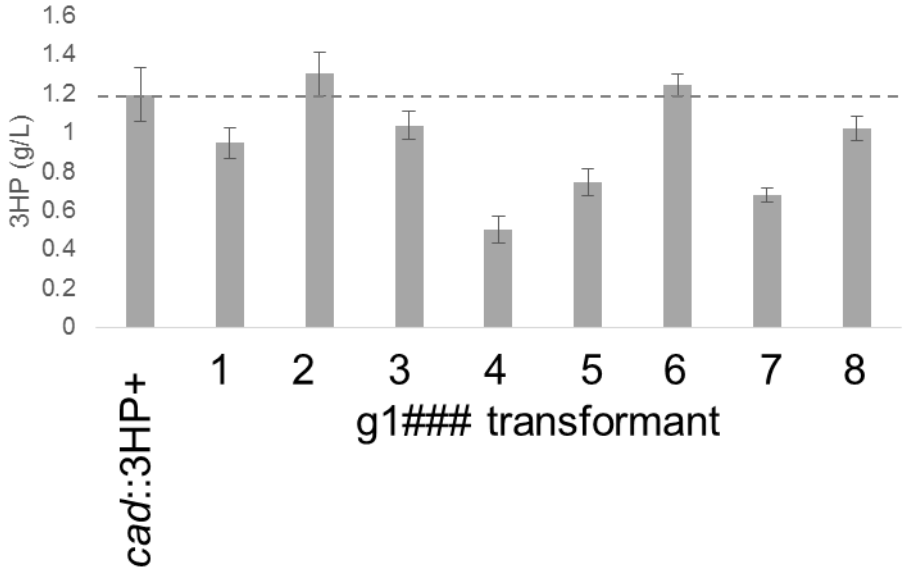



ANN trained on 3HP-responsive proteins is good (PCC=0.83) at predicting 3HP, and poor predictor of all other phenotypes.

68 3HP dose responsive proteins were knocked out and overexpressed *in silico* to predict the effect on 3HP production.

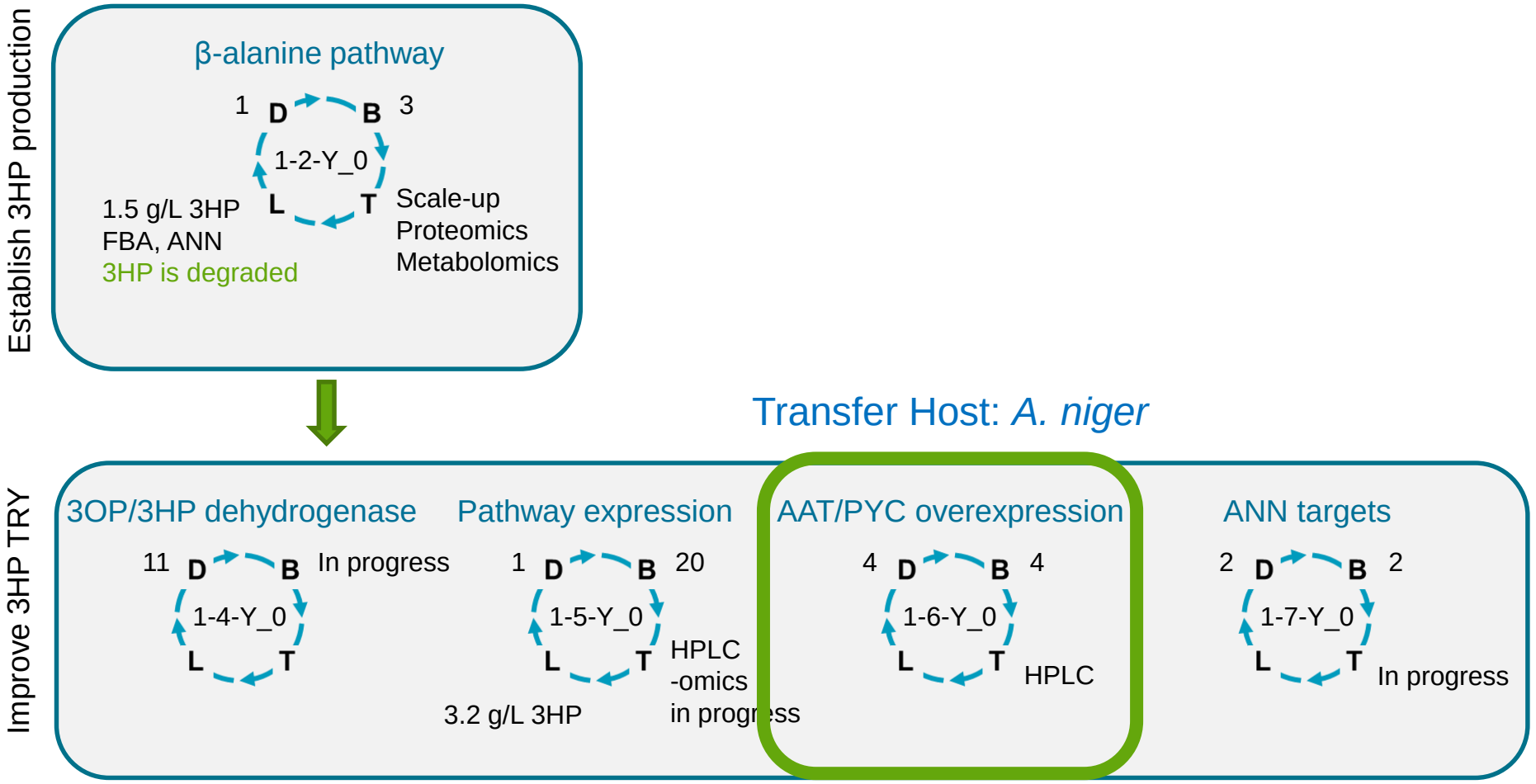
G9### (involved in cytoskeleton) deletion → 1.1x 3HP
no transformants obtained, likely essential gene

G1### (MFS transporter) overexpression → 1.4x 3HP
Graph below: no transformants with significantly improved 3HP



# 3HP DBTL cycles in progress



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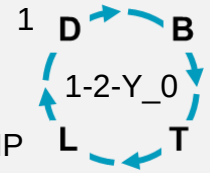
# 9-1-Y_0 Design, Build: 3HP host transfer

*Aspergillus* team is working on *A. niger*

Also working with *Rhodosporidium* team on transferring 3HP pathways into *R. toruloides*

Establish 3HP production

β-alanine pathway

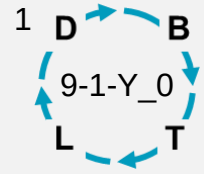



1.5 g/L 3HP
ANN, FBA

Scale-up
Proteomics
Metabolomics

Transfer 3HP pathway to *Aspergillus niger*

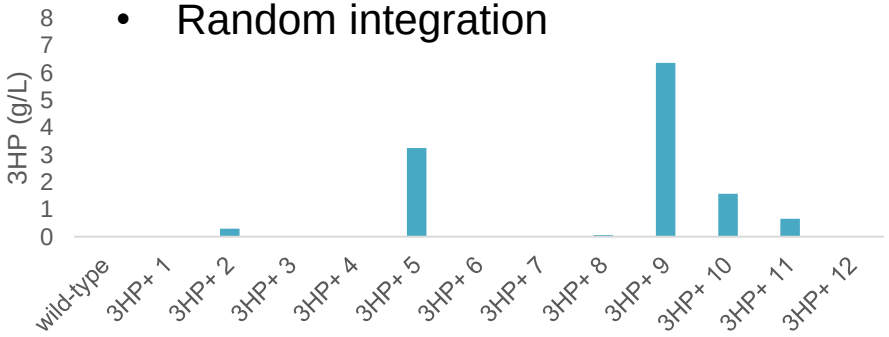
β-alanine pathway in *A. niger*

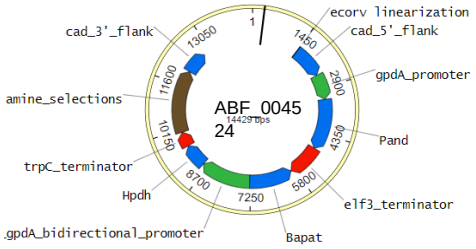


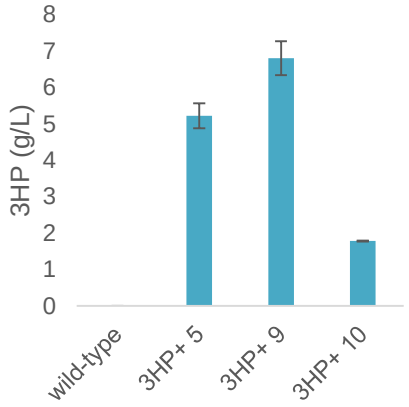

In progress
(32 samples)

*Aspergillus niger* transformant screening

- Random integration




- Random integration strains
- Different titers



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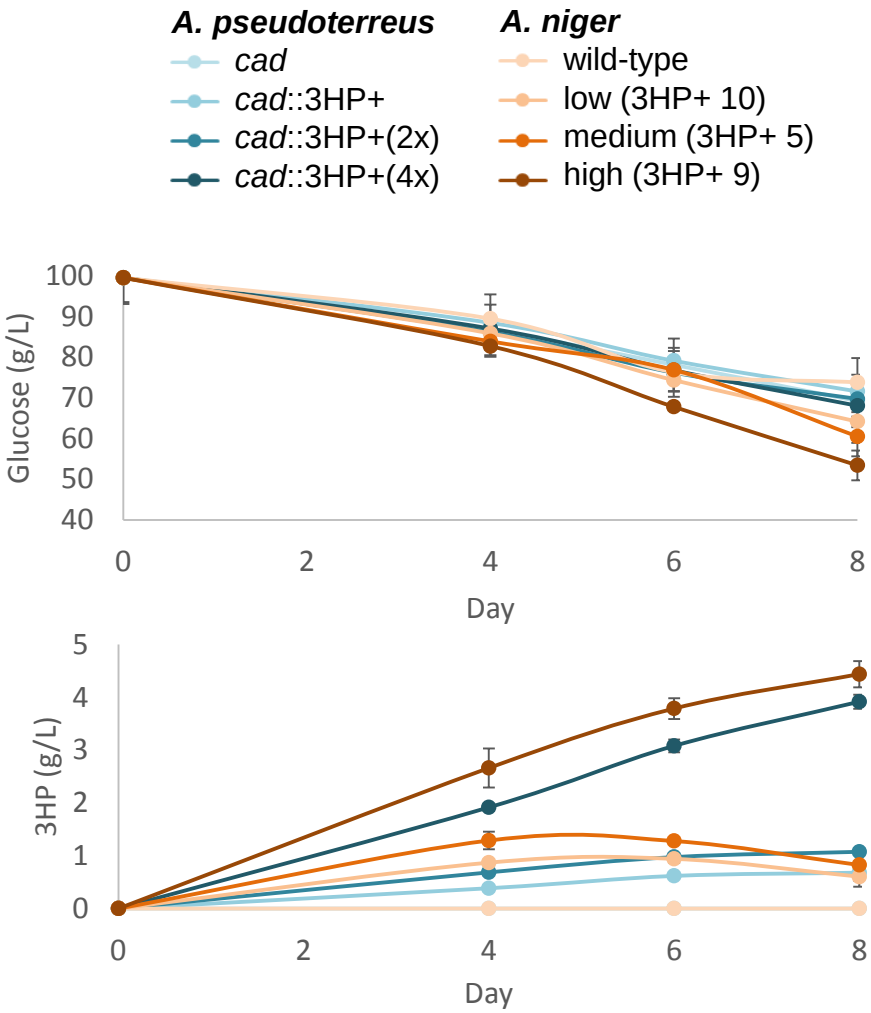
# 1-5-Y_0/9-1-Y_0 Test: 3HP host transfer




**TEST**, **LEARN** for
*Aspergillus* 3HP productivity comparison

Day 4 (8 strains x 4 reps = 32 samples)
- Production phase
- Prior to 3HP decrease

Targeted proteomics and intracellular metabolomics
- Enzyme concentration versus productivity in both species
- Rate limiting steps?

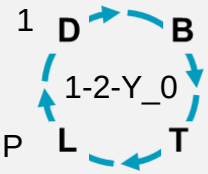
Extracellular metabolomics
- Product identification
- Flux modeling

Transcriptomics
- Identification of regulatory and nonintuitive targets

# 9-2-Y_0 Design, Build, Test: 3HP host transfer

Establish 3HP production

β-alanine pathway

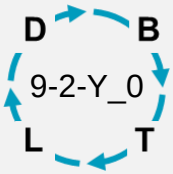



1.5 g/L 3HP
FBA, ANN
3HP is degraded

Scale-up
Proteomics
Metabolomics

Improve 3HP TRY

AAT/PYC overexpression

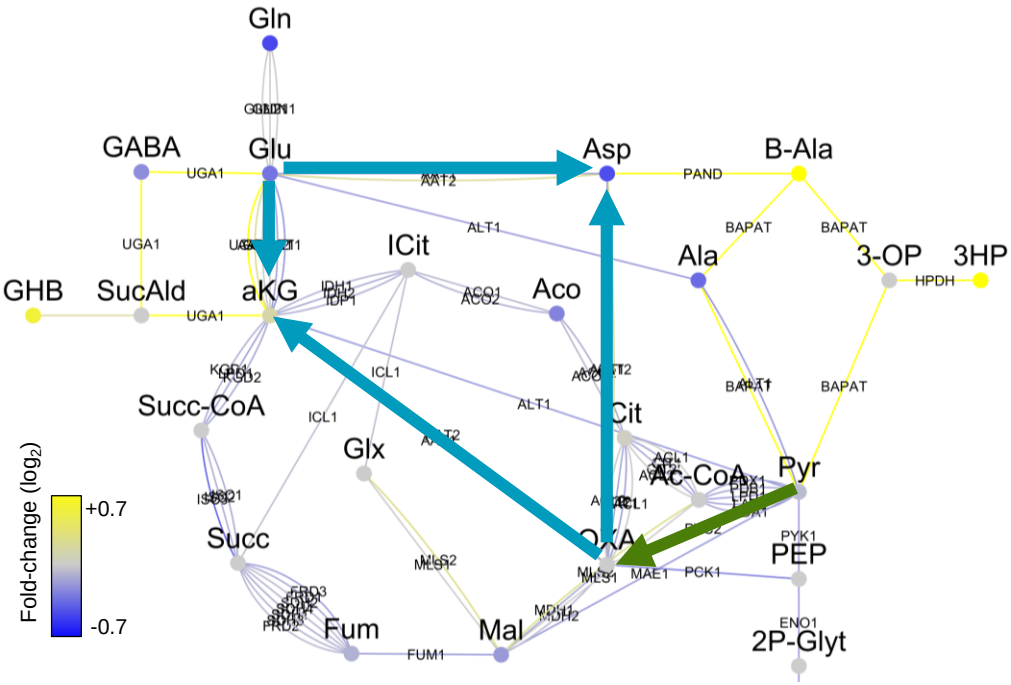



HPLC

## Overexpress β-alanine pathway precursor genes

**Aspartate Aminotransferase (AAT)**
- flux toward aspartate

**Pyruvate Carboxylase (PYC)**
- circumvent flux toward acetyl-CoA
- Incorporate $CO_2$ (increase yield)



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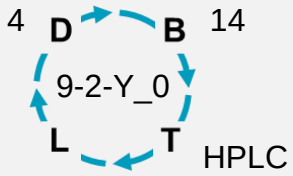
# 9-2-Y_0 Design, Build, Test: 3HP host transfer

AAT/PYC overexpression

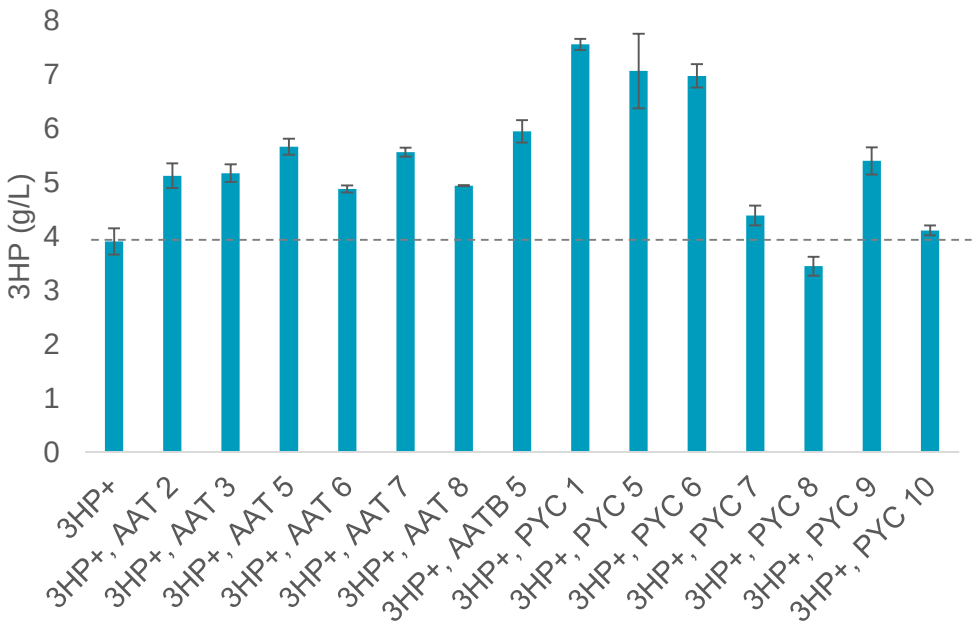



Improved 3HP

Aspartate aminotransferase (AAT)
transformants with significantly higher 3HP

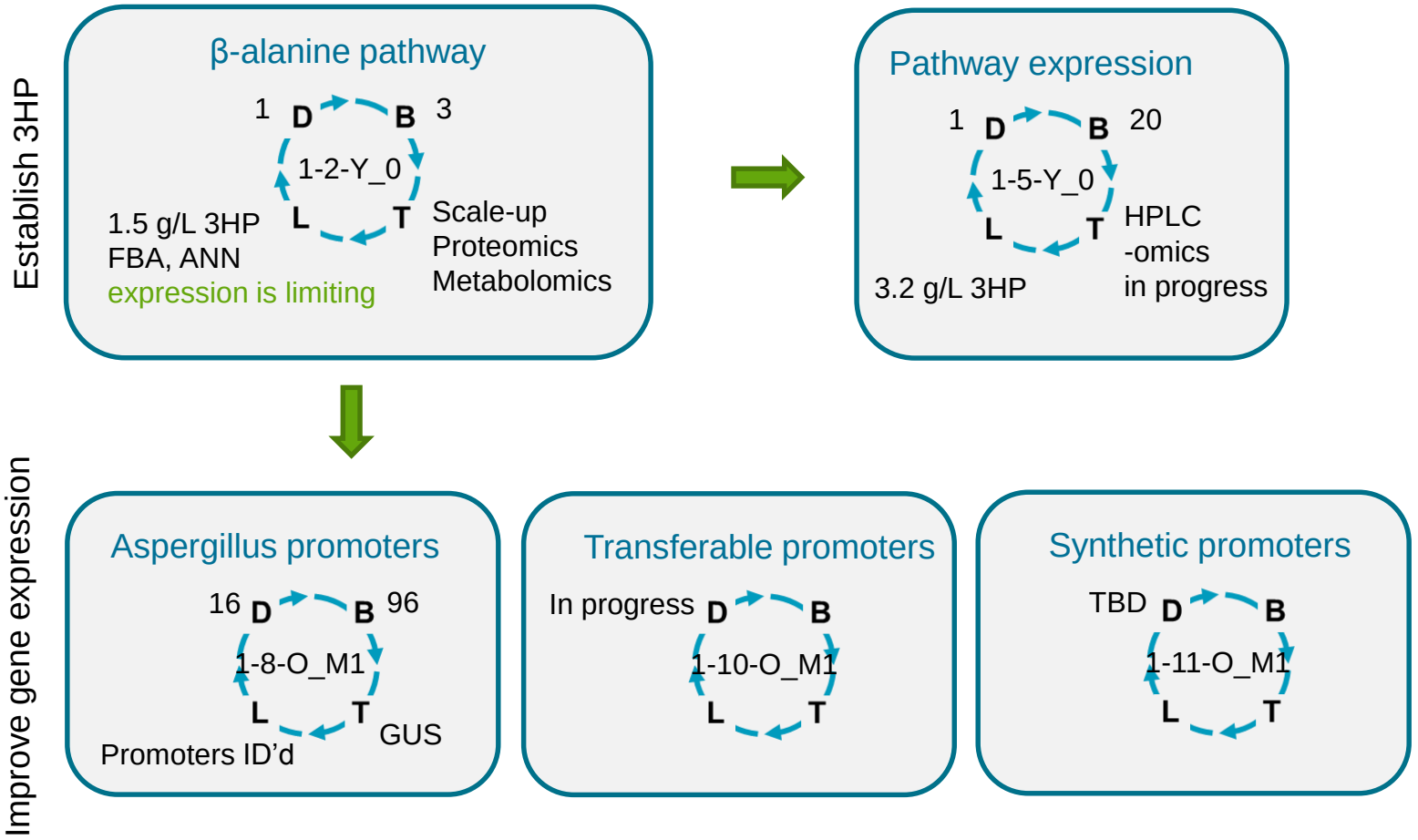
Pyruvate carboxylase (PYC)
transformants with significantly higher 3HP

*Aspergillus niger* transformant screening



# 1-8-O_M1 Design: high expression promoters



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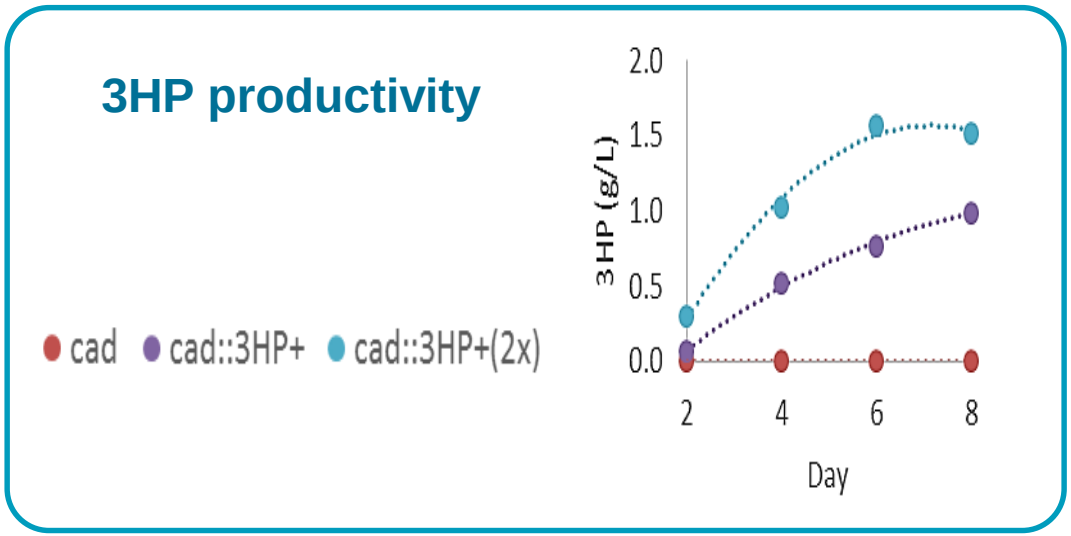
# Copy Number Correlates with Productivity



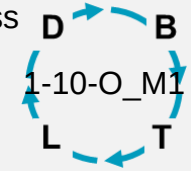

- Indicates gene **expression is limiting** in current 3 gene construct
- It would be great to be able to drive increased expression with **many strong promoters** available in our tool box

# 1-10-O_M1 Design: transferable promoters

Identify transferable promoters for fungi

Transferable promoters

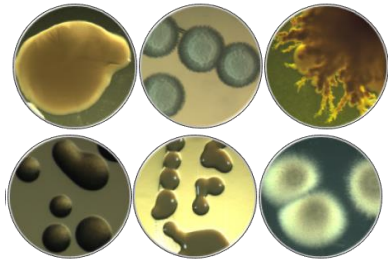
In progress




high expression promoters + vector with resistance gene

promoter library

**Goal**:
Identify promoter:selectable marker (*nat*) combinations usable across broad phylogenetic distances



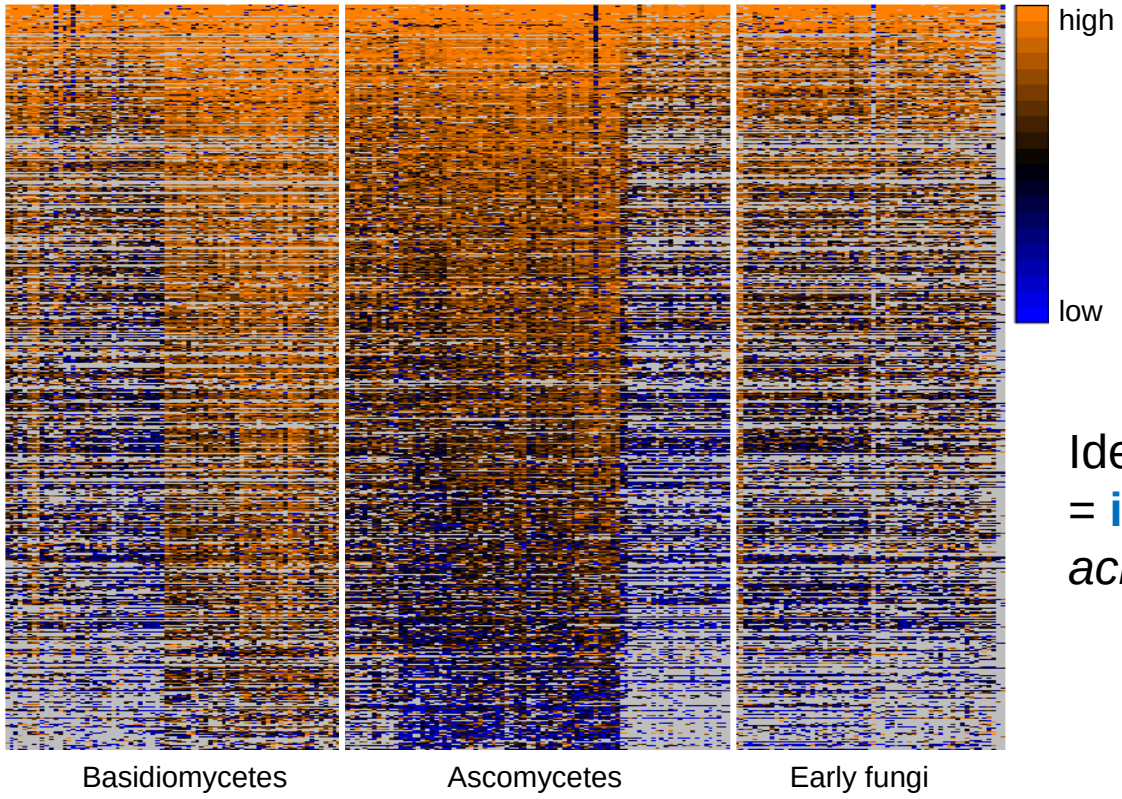
Transformation into diverse fungi

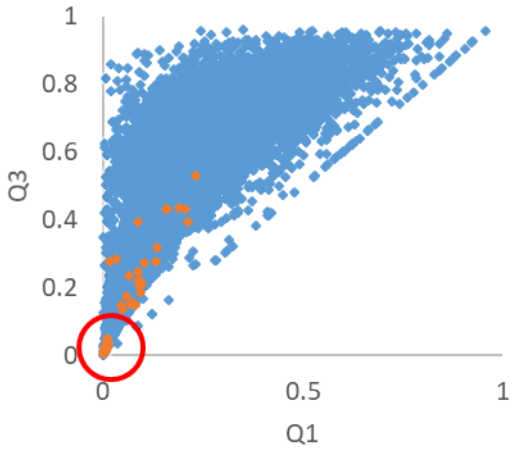
| Ascomycetes | Basidiomycetes |
|---|---|
| *Yarrowia lipolytica* | *Rhodosporidium toruloides* |
| *Zygoascus hellenicus* | *Trichosporon chiarellii* |
| *Lipomyces starkeyi* | *Fibulobasidium inconspicuum* |

select for antibiotic resistance sequence

# 1-10-O_M1 Design: transferable promoters

Gene expression level (order)



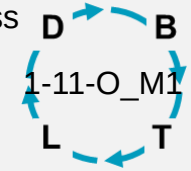

Identify highly expressed genes = **identify strong promoters**, *across* the Kingdom Fungi

# 1-11-O_M1 Design: synthetic promoters

High expression transferable synthetic promoters

Synthetic promoters

In progress



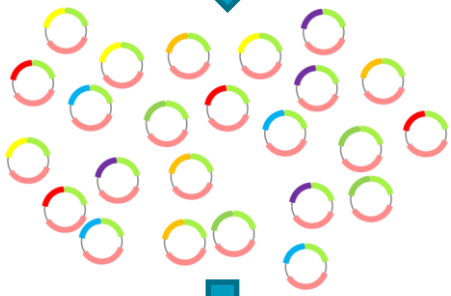

1-11-O_M1 Goal:
Quantify expression level of baseline and synthetic promoters in diverse fungi. Identify promoters with high expression level that are maintained across phylogenetic distance.

high expression and synthetic promoters

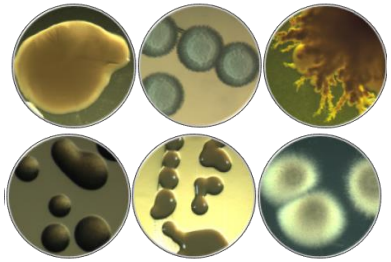
+

universal vector with fluorescent gene

promoter library



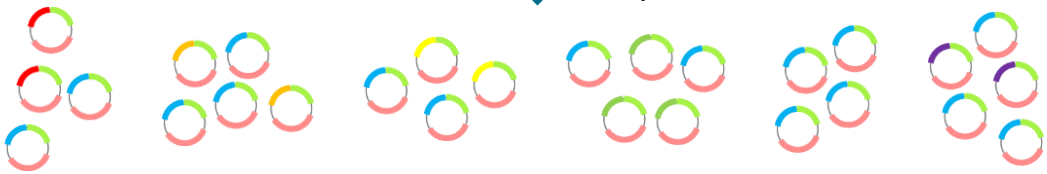
Transformation into diverse fungi



diverse DMR converting yeasts

| Ascomycetes | Basidiomycetes |
|---|---|
| *Yarrowia lipolytica* | *Rhodosporidium toruloides* |
| *Zygoascus hellenicus* | *Trichosporon chiarellii* |
| *Lipomyces starkeyi* | *Fibulobasidium inconspicuum* |

flow cytometry sort sequence





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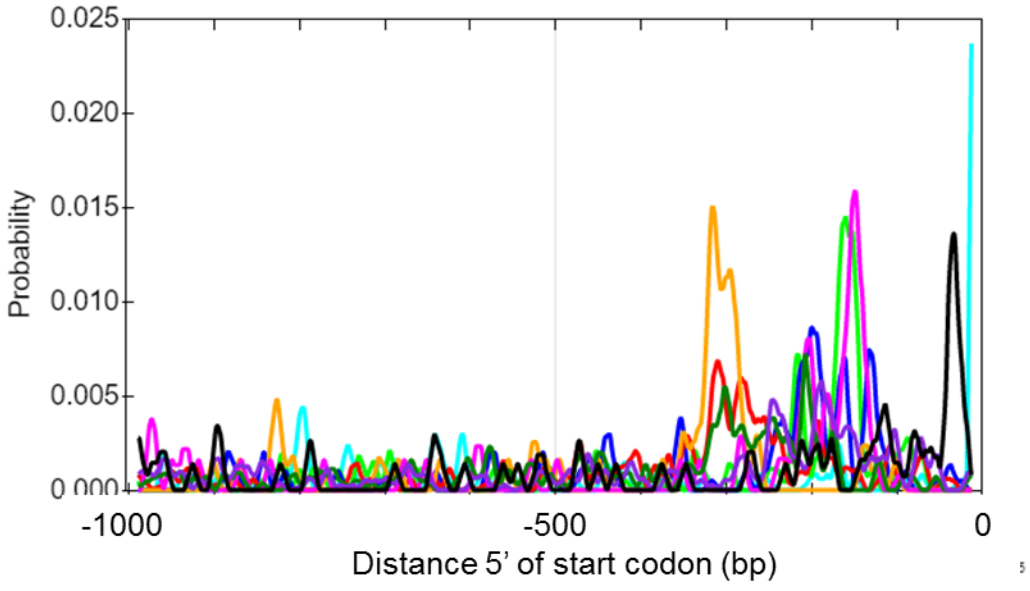
# 1-11-O_M1 Design: synthetic promoters

Positional motif enrichment in *geneX* promoters across fungi




| Motif | Most similar TF binding site (*S. cerevisiae*) |
|---|---|
| | Abc1 |
| | Abc2 |
| | Abc3 |
| | Abc4 |
| | Abc5 |
| | Abc6 |
| | Abc7 |
| | Abc8 |
| | Abc9 |
| | Abc10 |
| | Abc11 |
| | Abc12 |
| | Abc13 |
| | Abc14 |

Highly enriched motifs

- Some have positional requirements: Abc1-9
- Some are position-independent: Abc10-14

# FY20+ plans

- 3HP (*A. pseudoterreus* and *A. niger*)
  - Multi-omics based test/learn on *A. pseudoterreus* (1-5-Y_0) and *A. niger* (9-1-Y_0) strains with improved 3HP yield
  - Build/test/learn on 3HP catabolic genes (1-4-Y_0) targets; transfer gene target to highest yield strains
  - Test/learn on *A. niger* AAT/PYC strains with high productivity (9-2-Y_0) and incorporation of both gene targets into single strain
- Fungal build tools
  - DBTL for transferable and synthetic expression constructs (1-10-O_M1 & 1-11-O_M1).
  - FY20: Develop in vitro CRISPR technique that can delete multiple genes at same time and can be applied to different Aspergillus spp.

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- Matt Sturtevant
- Deepti Tanjore
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- Jeremy Zucker




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