

# RB-TnSeq elucidates dicarboxylic-acid-specific catabolism in $\beta$ -proteobacteria for improved plastic monomer upcycling

## Background/Objective:

- As components of polymers and plastics, dicarboxylic acids are a target for engineered microbial degradation and sustainable bioproduction
- We aimed to generate functional evidence for the genetic basis of dicarboxylic and fatty acid metabolism in four  $\beta$ -proteobacteria

## Approach:

- We identified four  $\beta$ -proteobacteria that displayed robust growth with dicarboxylic acid sole carbon sources and cultured their mutant libraries with dicarboxylic and fatty acids with carbon chain lengths from C3 to C12
- We identified  $\beta$ -oxidation genes, transcriptional regulators, and transporters with strong fitness phenotypes related to dicarboxylic acid utilization

## Results:

- The fitness data suggested different sets of  $\beta$ -oxidation genes are required for catabolizing dicarboxylic versus fatty acids of the same carbon chain lengths
- In *Ralstonia* CL21, we deleted two transcriptional repressors to improve its utilization of short chain dicarboxylic acids
- We engineered this R. CL21 to upcycle a mock mix of the dicarboxylic acids produced when polyethylene is oxidized, producing  $0.56 \pm 0.02$  g/L indigoidine

## Significance/Impacts:

- We demonstrated the potential of R. CL21 to upcycle plastics waste to products derived from tricarboxylic acid (TCA) cycle intermediates
- Our dataset is the most comprehensive study of dicarboxylic acid catabolism to date

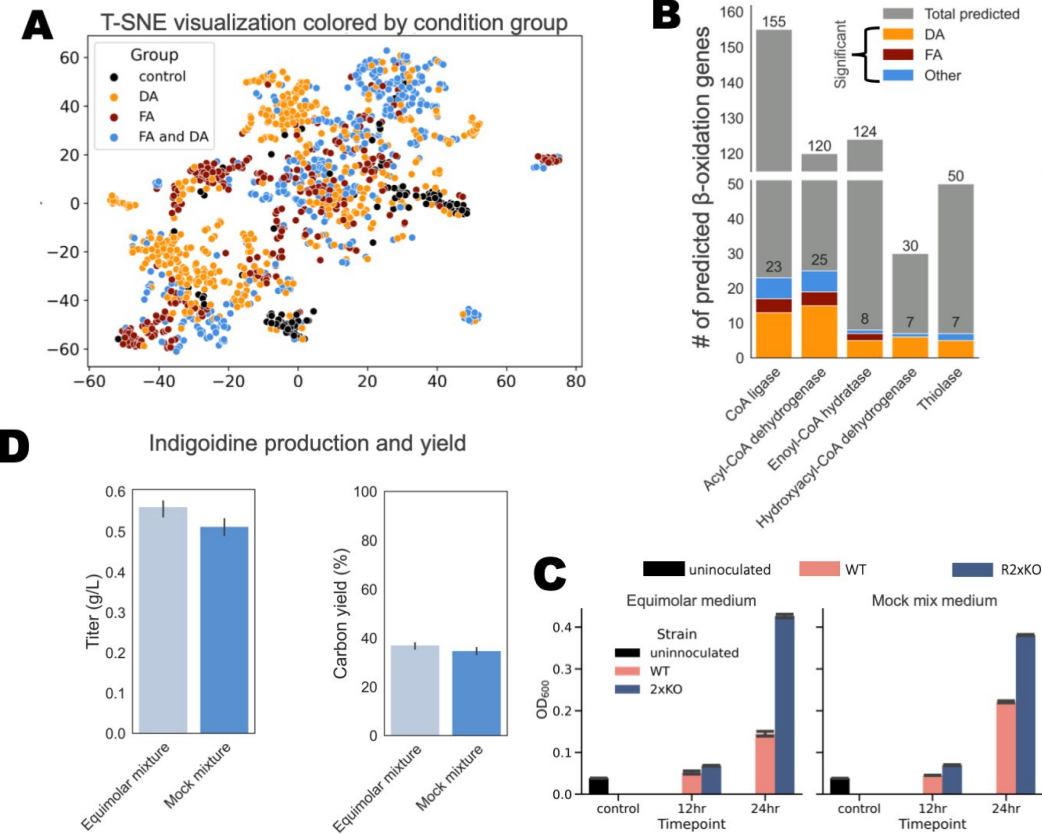


Figure caption: A) Genes with a significant fitness phenotype across four  $\beta$ -proteobacteria, plotted by dimensionality reduction of their fitness profile via t-sne. B) Total number of predicted  $\beta$ -oxidation genes across all four  $\beta$ -proteobacteria.  $\beta$ -oxidation genes with significant fitness are colored according to the conditions that elicited significant phenotypes: dicarboxylic acids only (orange), fatty acids only (red), or fatty/dicarboxylic acids and/or the control conditions (blue) C) Growth (as determined by OD<sub>600</sub> measurements) of R. CL21 WT and R2xKO over the course of the 24 hour consumption experiments. D) Plasmid-based production and yield of indigoidine in R2xKO from a mixture of dicarboxylic acids, measured colorimetrically

# Differences in GenBank and RefSeq annotations may affect genomics data interpretation for *Pseudomonas putida* KT2440

## Background

- Genomic information present in online knowledge bases is often used to make sense of data or to inform experimental decisions
- However, different databases may present conflicting information for the same genes and this is often not fully appreciated

## Approach

- Gene annotations for the *P. putida* KT2440 reference genomes deposited on RefSeq and GenBank were compared and used for the discovery of differentially expressed genes using a standard RNA-seq analysis pipeline

## Results

- 16% of genes under equivalent locus tag codes across GenBank and RefSeq were found to be annotated with different start/end positions
- These differences can be sufficient to significantly affect the interpretation of results derived from the analysis of RNA-seq datasets

## Significance

- This work provides a quantitative assessment of the differences in widely used genomics resources, promoting awareness and strengthening reproducibility and transparency in *P. putida* research

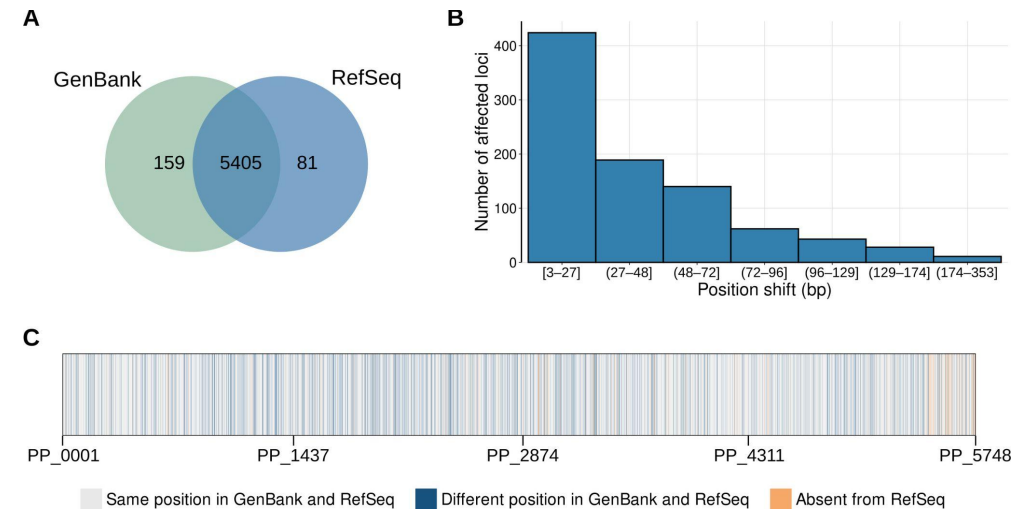


Figure caption: Comparison between features in GenBank and RefSeq *P. putida* KT2440 annotations. (A) Venn diagram comparing the number of features (genes) in GenBank (green) and RefSeq (blue) with the “protein-coding” descriptor. (B) Position differences in the start or end nucleotide of the ORFs annotated under equivalent locus tag codes (in base pairs). (C) Categorization of genes across all of the possible GenBank loci in comparison to their RefSeq counterpart. For these comparisons, RefSeq re-annotation locus tag codes were matched to the GenBank locus tag codes by using the “old\_locus\_tag” field whenever available. Please note that panel C does not accurately reflect the genome structure of *P. putida* KT2440, as locus tag codes do not necessarily match their relative genomic positions.

# Bioconversion of self-neutralized chemically depolymerized lignin streams into polyhydroxyalkanoates

## Background/Objective

- Lignin, a major component of biomass, remains underutilized due to its complex and recalcitrant structure
- Chemical depolymerization converts lignin into smaller, soluble molecules that can serve as potential carbon sources for microbial bioconversion

## Approach

- Chemically depolymerized lignin (CDL) was evaluated as a microbial substrate using both native and engineered PHA-producing strains
- Growth and PHA production were compared across aliphatic, aromatic, and mixed CDL streams to identify optimal conditions

## Results

- Both native and engineered microbes grew efficiently on CDL and produced diverse PHAs
- Native strains favored high-value MCL-PHAs, while engineered strains achieved higher yields; mixed CDL streams provided the best overall growth and production

## Significance/Impacts

- Demonstrates a sustainable pathway for transforming lignin waste into value-added bioplastics
- Establishes a foundation for cost-competitive biomanufacturing and improved lignin valorization through integrated chemical and biological conversion

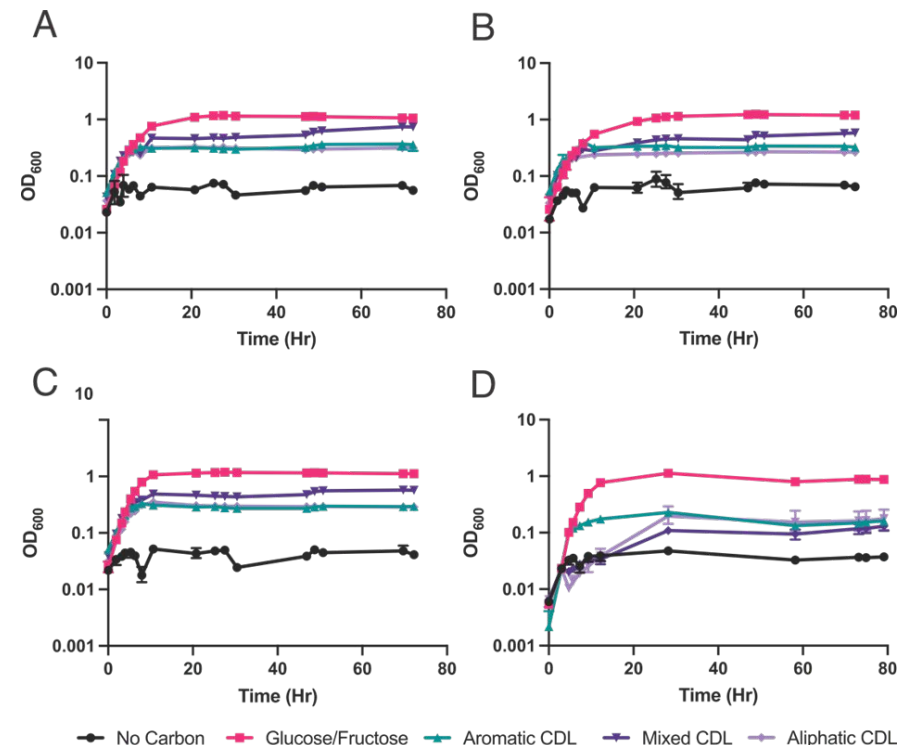


Figure caption: Growth curve of native and engineered PHA producing strains on CDL as the sole carbon source in minimal media. Black line represents inoculation in minimal media with no carbon source. Pink line represents growth on minimal media with a preferred carbon source, glucose or fructose (*C. necator* H16). Teal represents growth on minimal media with the aromatic CDL as the sole carbon source. Dark purple represents growth on minimal media with the mixed CDL as the sole carbon source. Light purple represents growth on minimal media with the aliphatic CDL as the sole carbon source. (A) *C. necator* H16, (B) *P. putida* KT2440, (C) *E. coli* LSBJ STQKAB, (D) *T. thermophilus* HB27. N=3.

# Enabled Publications

# The oleaginous yeast *Rhodospiridium toruloides* engineered for biomass hydrolysate-derived (E)- $\alpha$ -bisabolene production

## Background/Objective

- Bisabolene, a naturally occurring terpene that, when saturated, is a suitable biodiesel, jet fuel alternative, and fuel additive
- We sought to increase titers beyond previous literature highs via unexplored strategies

## Approach

- We overexpressed the entire 8-gene mevalonate pathway from *S. cerevisiae* into a high-copy bisabolene synthase parent strain
- We collected proteomics and metabolomics to identify potential bottlenecks

## Results

- The best strain yielded 20.8 g/L bisabolene from 300 g/L total sugars corn stover hydrolysate
- Multi-omics of the best strain revealed one to three potential bottlenecks within the production pathway

## Significance/Impacts

- The highest bisabolene titer and productivity in the literature were achieved
- This work demonstrates that much higher titers can be achieved if identified bottlenecks can be resolved, paving the way to commercially relevant titers

Adamczyk, P., et.al. Metabolic engineering. doi: 10.1016/j.ymben.2025.02.014(JBEI #122)

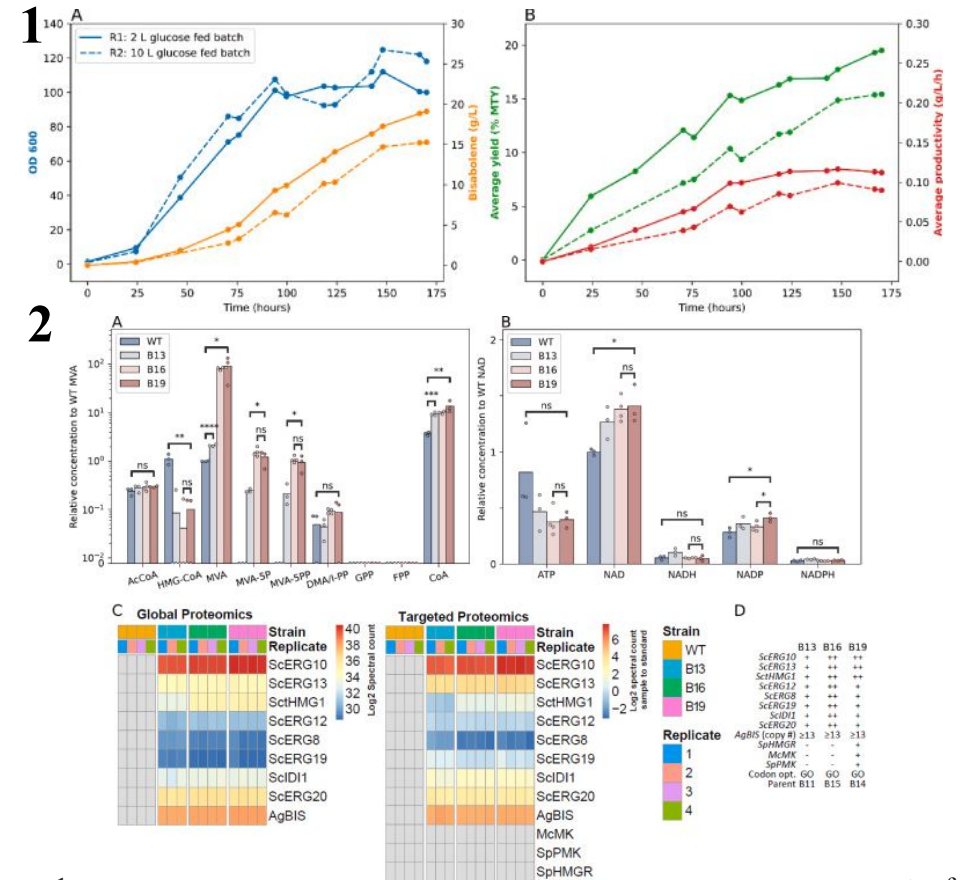


Figure 1A,B Time-course key performances of glucose fed-batch bioreactor experiments of strain B22. Bioreactor runs with at 2 L and 10 L scales with an identical defined glucose medium feeding strategy starting with a minimal medium base recipe (R1 and R2, respectively) Figure 2A-D Relative intracellular concentrations of MVAP intermediates, ATP, and cofactors on DMR across different engineered strains vs WT. All concentrations in (2A,B) are normalized to the average of WT MVA and NAD, respectively. 2C Global and targeted proteomics of the heterologous *S. cerevisiae* MVAP. Heat maps indicate log2 intensity and log2 ratio of sample to standard intensity, respectively.

# Inducible flippase-mediated metabolic engineering of *Rhodospiridium toruloides* for enhanced 3-hydroxypropionic acid production from corn stover hydrolysate

## Background/Objective

- *R. toruloides* is a promising host for utilizing lignocellulosic biomass, but bioproduct yield enhancement is limited by the lack of robust genetic tools
- Objective: Develop a marker-free inducible FLP-FRT system for iterative genome editing to optimize 3HP production

## Approach

- Established synthetic inducible promoters for tight FLP control, enabling highly efficient antibiotic marker removal
- Used the FLP-FRT system for four rounds of iterative editing, focusing on optimizing the 3HP pathway via acetyl-CoA/malonyl-CoA precursor supply

## Results

- Tightly regulated synthetic promoters exhibited low basal fluorescence and a 10.1-fold dynamic range
- The best strain achieved a maximum 3HP titer of 69.4 g/L in fed-batch fermentation from corn stover hydrolysate medium

## Significance/Impacts

- The 69.4 g/L 3HP titer is the highest reported in yeast from lignocellulosic hydrolysate, validating *R. toruloides* as a robust production platform
- The inducible FLP-FRT system is a tightly controlled, robust platform that simplifies and enables repeated genetic modification

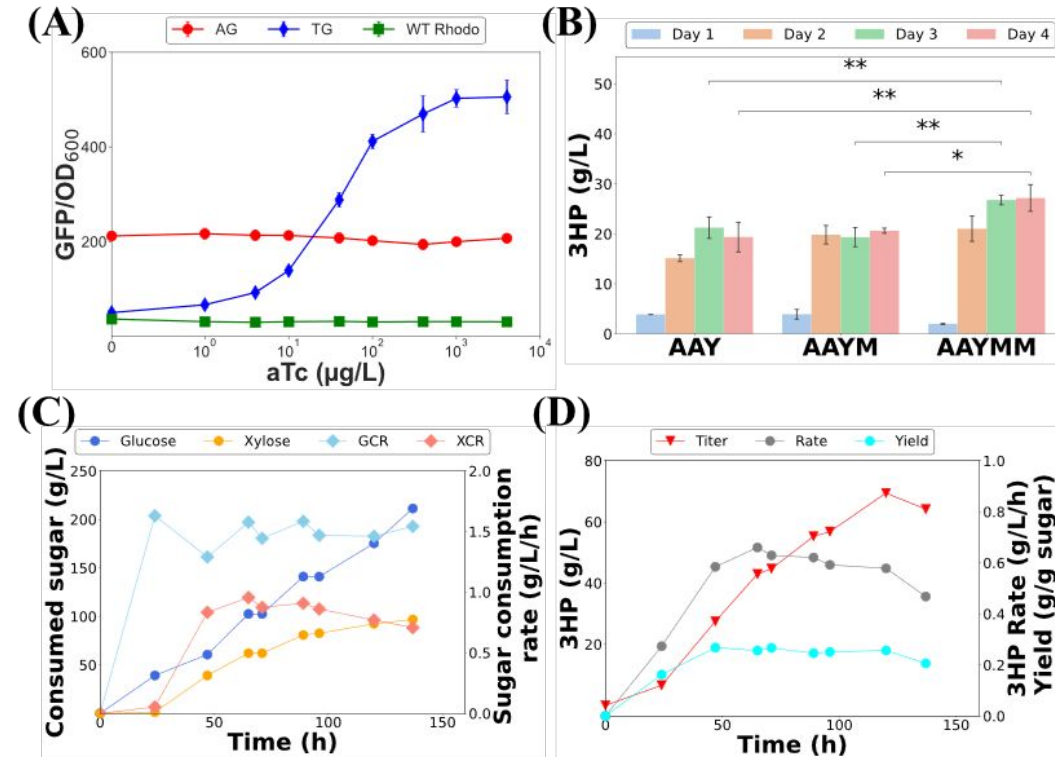


Figure caption: (A) The synthetic *ANT1tetO* promoter exhibits tight regulation and a 10.1-fold dynamic range in response to the inducer aTc. (B) 3HP titer profiles show the AAYMM strain (best strain) reaching 27.2 g/L on day 4 in shake flask cultures, a 32% increase over the AAYM strain. (C-D) Fed-batch fermentation profiles of the best strain in DMR8UmY medium show enhanced concurrent utilization of glucose and xylose (reaching a maximum xylose consumption rate of 1.0 g/L/h) and achieved the peak 3HP titer of 69.4 g/L at 120 h.