Developing synthetic biology tools to produce muconate from lignin-derived aromatic compounds using *Rhodococcus opacus*

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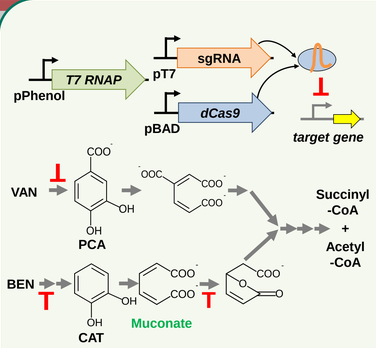

Adipic acid, a monomer for nylon production, is currently produced from petroleum derivatives, requiring an alternative process for its sustainable production. Muconate can be converted into various chemicals, including adipic acid. Using non-model organisms, multiple labs have demonstrated muconate production from lignin-derived aromatic compounds, with glucose used as a growth substrate. *Rhodococcus opacus* is well suited for valorizing lignin (1-8), but developing this promising chassis had been challenging due to limited genetic engineering tools. To address this issue, we have developed various synthetic biology tools (9-12), including a gene repression system based on CRISPR interference (CRISPRi) and a knockout method. In this presentation (13), we discuss our CRISPRi tool's utility by demonstrating the inducible accumulation of muconate from aromatics. Additionally, its inducibility and partial repressibility are discussed compared to gene knockout's complete metabolic flux blocking. We also provide a cloning strategy that enables constructing multiple CRISPRi plasmids without any PCR step, facilitating this GC-rich organism's engineering. Our tools will be useful to engineer this chassis for lignin conversion into plastic monomers.

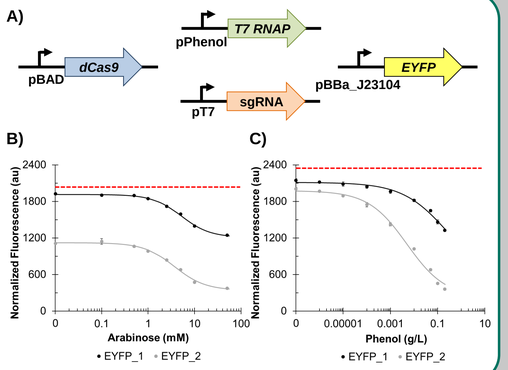

Figure 1. T7 RNAP-based CRISPRi of EYFP in *R. opacus*. The T7 RNAP-based CRISPRi system was used to repress a constitutively expressed *EYFP* gene cassette integrated into the previously identified neutral site ROCI2. **A)** Schematic of genetic constructs. Normalized fluorescence transfer curves were generated in response to **B)** arabinose (0.2g/L phenol) and **C)** phenol (50mM arabinose). Induction of dCas9 via arabinose (B) resulted in 5-39% repression via sgRNA EYFP_1 and 45-82% via sgRNA EYFP_2, relative to the no-sgRNA control when induced with 0.2 g/L phenol (red line). Induction of the T7 RNAP via phenol (C) resulted in 9-44% and 15-85% repression by sgRNAs EYFP_1 and EYFP_2, respectively, relative to the no-sgRNA control when induced with 50 mM arabinose (red line). Values are averages of three replicates, with error bars representing one standard deviation; solid lines represent fitted curves.

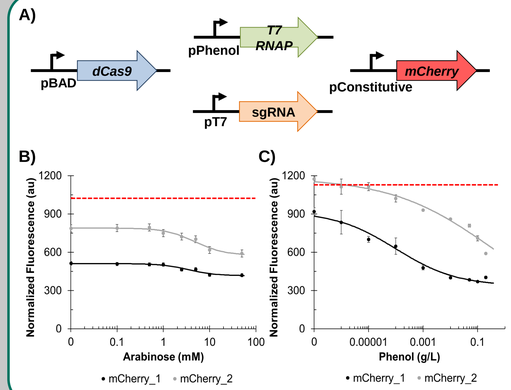

Figure 2. T7 RNAP-based CRISPRi of mCherry in *R. opacus*. The T7 RNAP-based CRISPRi system was used to repress a constitutively expressed *mCherry* gene cassette integrated into the previously identified neutral site ROP8I. **A)** Schematic of genetic constructs. Normalized fluorescence transfer curves were generated in response to **B)** arabinose (0.2 g/L phenol) and **C)** phenol (50mM arabinose). Induction of dCas9 via arabinose (B) resulted in 50-59% repression via sgRNA mCherry_1 and 23-43% via sgRNA mCherry_2, relative to the no-sgRNA control when induced with 0.2 g/L phenol (red line). Induction of the T7 RNAP via phenol (C) resulted in 19-64% and -4-48% repression by sgRNAs mCherry_1 and mCherry_2, respectively, relative to the no-sgRNA control when induced with 50 mM arabinose (red line). Values are averages of three replicates, with error bars representing one standard deviation; solid lines represent fitted curves.

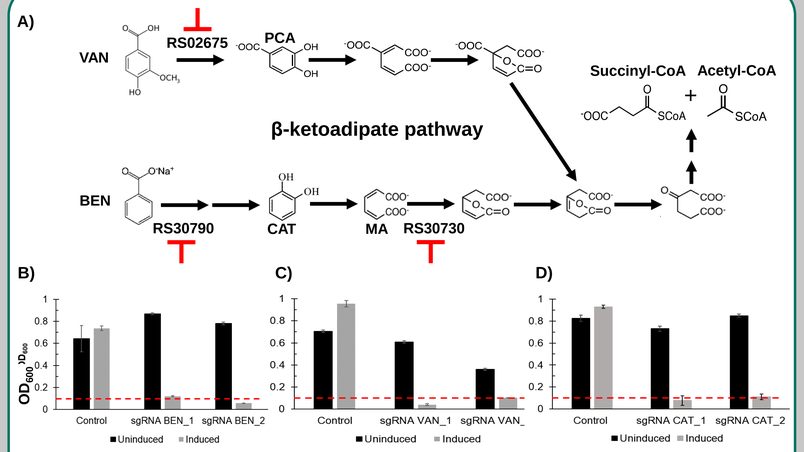

Figure 3. T7 RNAP-based CRISPRi of aromatic catabolism genes in *R. opacus*. The T7 RNAP-based CRISPRi system was used to repress aromatic degradation genes identified by RNA-Seq to be related to the consumption of benzoate (BEN; RS30790), vanillate (VAN; RS02675), and catechol (CAT; RS30730). **A)** Pathway schematic detailing the genes targeted for repression (PCA, protocatechuate; MA, muconate). For all experiments (B-D), the uninduced condition is 0 g/L phenol and 0 mM arabinose, while the induced condition is 0.2 g/L phenol and 50 mM arabinose. **B)** Strains expressing no sgRNA (Control), sgRNA BEN_1, or sgRNA BEN_2 were grown on 1 g/L sodium benzoate and either uninduced or induced conditions for 48 hours; the final cell culture density (OD_{600}) is depicted. **C)** Strains expressing no sgRNA (Control), sgRNA VAN_1, or sgRNA VAN_2 were grown on 0.75 g/L vanillate and either uninduced or induced conditions for 48 hours; the final OD_{600} value is depicted. **D)** Strains expressing no sgRNA (Control), sgRNA CAT_1, or sgRNA CAT_2 were grown on 1 g/L sodium benzoate (degraded via catechol pathway) and either uninduced or induced conditions for 40 hours; the final OD_{600} value is depicted. The red dashed lines represents the initial OD_{600} of 0.1. Bars are averages of three replicates, and error bars represent one standard deviation.

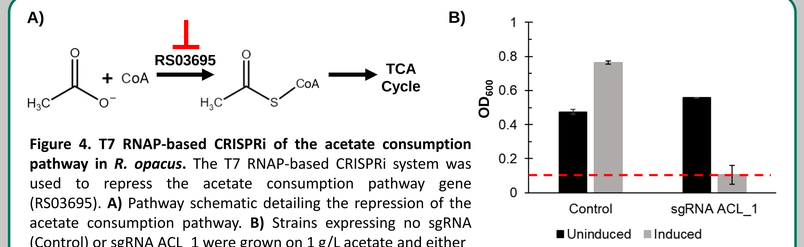

Figure 4. T7 RNAP-based CRISPRi of the acetate consumption pathway in *R. opacus*. The T7 RNAP-based CRISPRi system was used to repress the acetate consumption pathway gene (RS03695). **A)** Pathway schematic detailing the repression of the acetate consumption pathway. **B)** Strains expressing no sgRNA (Control) or sgRNA ACL_1 were grown on 1 g/L acetate and either uninduced (0 g/L phenol and 0 mM arabinose) or induced (0 g/L phenol and 0 mM arabinose) conditions for 72 hours; the final cell culture density (OD_{600}) is depicted. The red dashed line represents the initial OD_{600} of 0.1. Bars are averages of three replicates, and error bars represent one standard deviation.

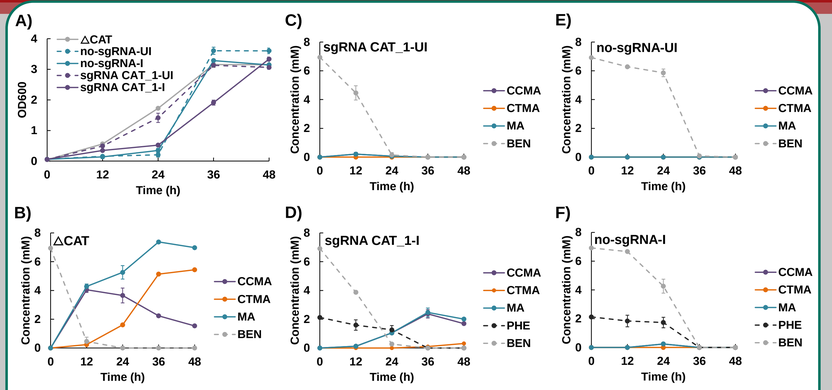

Figure 5. Muconic acid production from 1 g/L benzoate with 4 g/L glucose. Muconic acid accumulation from benzoate (BEN) was enabled by knocking out (ΔCAT) or knocking down (sgRNA CAT_1) muconate cycloisomerase (RS30730 in Fig. 3). Uninduced (UI; 0 g/L phenol and 0 mM arabinose) cultures were compared with induced (I; 0.2 g/L phenol and 50 mM arabinose) cultures, the ΔCAT strain, and the no-sgRNA control (uninduced and induced). **A)** Growth measured over time. **B-F)** Concentrations of *cis,cis*-muconate (CCMA), *cis,trans*-muconate (CTMA), total muconate (MA), PHE, and BEN over time for the indicated strain. Data points represent the mean of triplicate cultures, with error bars indicating the standard deviation.

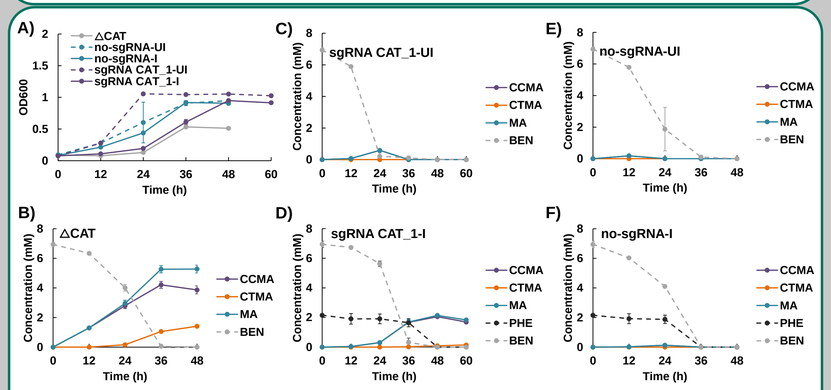

Figure 6. Muconic acid production from 1 g/L benzoate with 0.657 g/L hydroxybenzoic acid. Muconic acid accumulation from benzoate (BEN) was enabled by knocking out (ΔCAT) or knocking down (sgRNA CAT_1) muconate cycloisomerase (RS30730 in Fig. 3). Uninduced (UI; 0 g/L phenol and 0 mM arabinose) cultures were compared with induced (I; 0.2 g/L phenol and 50 mM arabinose) cultures, the ΔCAT strain, and the no-sgRNA control (uninduced and induced). **A)** Growth measured over time. **B-F)** Concentrations of *cis,cis*-muconate (CCMA), *cis,trans*-muconate (CTMA), total muconate (MA), PHE, and BEN over time for the indicated strain. Data points represent the mean of triplicate cultures, with error bars indicating the standard deviation.

1. Chatterjee A, DeLorenzo DM, Carr R, Moon TS. 2020. *Current opinion in biotechnology* 64:10-6
2. Davis K, Moon TS. 2020. *Current opinion in chemical biology* 59:23-9
3. Henson WR et al. 2018. *Metabolic engineering* 49:69-83
4. Yoneda A et al. 2016. *Nucleic Acids Res.* 44:2240–54
5. Henson WR, Hsu F-F, Dantas G, Moon TS, Foston M. 2018. *Biotechnology for biofuels* 11:339
6. Roell GW et al. 2019. *Metabolic engineering* 55:120-30
7. Hollinshead WD, Henson WR, Abernathy M, Moon TS, Tang YJ. 2016. *Biotechnology and bioengineering* 113:91-100
8. Anthony WE et al. 2019. *Biotechnology for biofuels* 12:192
9. DeLorenzo DM, Henson WR, Moon TS. 2017. *ACS Synthetic Biology* 6:1973–8
10. DeLorenzo DM, Moon TS. 2018. *Scientific reports* 8:6019
11. DeLorenzo DM, Moon TS. 2019. *ACS Synthetic Biology* 8:1921-30
12. DeLorenzo DM, Rottinghaus AG, Henson WR, Moon TS. 2018. *ACS Synthetic Biology* 7:727-38
13. DeLorenzo DM, Diao J, Carr R, Hu Y, Moon TS. ACS Synthetic Biology. doi.org/10.1021/acssynbio.0c00591