Development of a Genetic Toolkit in *Rhodococcus opacus* PD630 for Lignin Valorization

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Project Goals: The overall goal of this project is to interrogate the metabolic networks and genetic regulation that control the utilization of and tolerance to thermochemically depolymerized lignin, focusing on phenolics, in *R. opacus*.

Lignocellulosic biomass is an abundant and renewable feedstock that can be converted into biofuels and commodity chemicals using microorganisms. An ideal organism for biomass conversion should possess capabilities for consumption of both cellulose-based sugars and toxic lignin-derived aromatic compounds. Additionally, a candidate microbe should be tolerant to growth inhibiting compounds generated during lignocellulose depolymerization, genetically tractable, and demonstrate a rapid growth rate. The Actinomycetales *Rhodococcus opacus* PD630 (*R. opacus*) is a gram-positive microbe, known for high levels of triacylglycerol accumulation, which meets these biomass conversion qualifications. To enhance its innate aromatic-degrading capacity, we applied adaptive evolution, a growth-based strain selection method, by sequentially sub-culturing cells in diverse combinations of lignin-derived aromatic compounds as sole carbon sources. Our adapted strains demonstrated higher growth rates and higher lipid accumulation compared to the wild type strain. Whole genome sequencing, RNA-seq, targeted metabolomics, and ^{13}C-fingerprinting analysis have identified possible aromatic tolerance and utilization mechanisms such as upregulation of degradation pathways and putative transporters for aromatic compounds.

Despite our increased understanding of aromatic tolerance and utilization mechanisms, few genetic elements and parts have been directly characterized in *R. opacus,* limiting its future

industrial applications. To enable *R. opacus* as a future chassis for biomass conversion, we have developed a suite of genetic tools for reliable and predictable gene expression: **1)** six fluorescent reporters in three distinct wavelength ranges for quantifying promoter output, **2)** a constitutive promoter library spanning a 45-fold change in fluorescence output, **3)** three chemically inducible promoters for tunable gene expression, **4)** a dynamic metabolite sensor that detects ammonium concentration, **5)** a collection of metabolite sensors that detect various aromatic compounds, and **6)** a recombinase-based system for integration of exogenous DNA into newly identified neutral sites within the genome. Additionally, a CRISPR interference system for targeted gene repression has been developed and a set of stable reference genes for RT-qPCR have been identified. Overall, this work expands the ability to control and characterize gene expression in *R. opacus* and is a critical first step towards future fuel and chemical production in this host.

Publications

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3. WD Hollinshead, WR Henson, M Abernathy, TS Moon and YJ Tang. Rapid Metabolic Analysis of *Rhodococcus opacus* PD630 via parallel ^{13}C-Metabolite Fingerprinting, *Biotechnol. Bioeng.* 113, 91-100 (2016)

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