Dynamic Control of Aromatic Catabolism, *In Situ* Efflux Pump Engineering, and High-Throughput Functional Genomics in *P. putdia* KT2440 Enabled by CRISPR-Cas9

Jacob Fenster*,[1,3] (jacob.fenster@colorado.edu), Allison Werner,[2,3] Emily Freed[1,3], Erica Prates[3], Andrew Levitt[1], Audrey Watson[1], Jeff Cameron[1], Carrie Eckert[1,2,3], and **Gerald A. Tuskan**[3]

[1]University of Colorado, Boulder [2]National Renewable Energy Laboratory, Golden, CO; and [3]Center for Bioenergy Innovation, Oak Ridge National Laboratory, Oak Ridge, TN

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The Center for Bioenergy Innovation (CBI) vision is *to accelerate domestication of bioenergy-relevant, non-model plants and microbes to enable high-impact innovations at multiple points in the bioenergy supply chain.* CBI has identified key barriers for the current bioeconomy in (1) high-yielding, robust feedstocks, (2) lower capital and processing costs via consolidated bioprocessing (CBP) to specialty biofuels, and (3) methods to create valuable byproducts from the lignin residues. CBI will identify and utilize key plant genes for growth, yield, composition and sustainability traits as a means of achieving lower feedstock costs, focusing on the perennial feedstocks - poplar and switchgrass. We will convert these feedstocks to specialty biofuels (C4 alcohols and esters) using CBP at high rates, titers and yield. CBP will be combined with cotreatment or pretreatment. CBI will maximize process and product value by *in planta* modifications of lignin and biological funneling of lignin to value-added chemicals. Techniques for rapid domestication of non-model microbes and plants will be improved.

Pseudomonas putida KT2440 is a promising chassis for the renewable conversion of lignin into commodity chemicals. While this organism has a wealth of genetic tools available, high-throughput (HTP) experiments that enable forward engineering have not yet been published in this organism. As current microbial engineering efforts rely on the Design, Build, Test cycle to generate desirable production phenotypes, it is critical to deploy HTP capabilities in this organism to domesticate this host. Towards this goal, this work seeks to leverage CRISPR-Cas9 technologies to demonstrate HTP capabilities on various biotechnologically relevant targets.

First, CRISPR-interference was optimized by screening inducible promoters expressing dCas9 on various targets. The arabinose inducible promoter was shown to be valuable for targeting essential genes such as *ftsZ* and *rpoD,* where a tightly regulated off-state allowed for the construction of inducible knockdown mutants. The lactose inducible system, with its higher induction strength was shown to be applicable for targeting metabolic proteins such as *pcaIJ* and *catB*. The CRISPRi system was then used to accumulate more 4.75 times more beta-ketoadipate in shake flask by inducing the knockdown of *pcaIJ*. This initial study demonstrating the usefulness of CRISPRi has laid the foundation for the study of a guide RNA (gRNA) library, constructed through a collaboration with JGI, targeting every coding region in the KT2440 genome and pilot experiments are showing promise that all 87,000 gRNAs will be able to be studied with high resolution.

Second, the CRISPR-Cas9 genome editing toolset first developed by Sun et al. (2018) has been optimized for library scale mutagenesis. The minimum homology length requirements for efficiently introducing both deletion and single nucleotide polymorphism (SNP) mutations was determined and the transformation protocol was optimized to increase the number of edited cells 100-fold while keeping the editing efficiency at 100%. This updated design was used to introduce SNPs in the TolC-like multidrug efflux pump, ttgABC, that were discovered during adaptive laboratory evolution. Efforts are underway to conduct deep scanning mutagenesis of this efflux pump in HTP to discover novel mutations that determine substrate binding and recognition important to both antibiotic resistance and tolerance to high lignin concentrations. Last, to make future genome editing experiments a plug and play effort, the activity of 150,000 gRNAs, constructed through a collaboration with JGI, will be assayed in order to catalogue efficient gRNAs and to develop a predictive gRNA model.

References/Publications

1. Sun J, Wang Q, Jiang Y, Wen Z, Yang L, Wu J, et al. Genome editing and transcriptional repression in Pseudomonas putida KT2440 via the type II CRISPR system. Microb Cell Fact. 2018 Mar 13;17(1):41

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