CRISPR-Tool Development in *Pseudomonas putida* KT2440 for High-Titer Strain Engineering and Multiplexed Approaches

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Project Goals: 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 will address strategic barriers to the current bioeconomy in the areas of: 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. CBI will identify and utilize key plant genes for growth, composition and sustainability phenotypes as a means of achieving lower feedstock costs, focusing on poplar and switchgrass. We will convert these feedstocks to specialty biofuels (C4 alcohols and C6 esters) using CBP at high rates, titers and yield in combination with cotreatment or pretreatment. CBI will maximize product value by *in planta* modifications and biological funneling of lignin to value-added chemicals.

The programmability of CRISPR-Cas machinery enables library based high-throughput and multiplexed experiments that have allowed for rapid genotype-phenotype mapping (Peters et al., 2016), protein engineering (Garst et al., 2017), strain engineering (Tarasava, Liu, Garst, & Gill, 2018), and gene discovery (Shalem et al., 2014). Both CRISPR-Cas editing and interference have been demonstrated in the bacteria *Pseudomonas putida* KT2440, a promising candidate for the industrial production of renewable chemicals from lignin. Although these studies establish the foundation for CRISPR-tool development in *P. putida*, the need remains for characterization and optimization of these tools before they can be leveraged for high-throughput and multiplexed experiments.

In this study, CRISPR-interference (CRISPRi) is optimized by screening inducible promoter systems that express catalytically-dead spCas9. The arabinose inducible promoter system performed best and was used to image the repression of the essential division protein ftsZ in real time. Future studies will quantify the dynamic range of repression by targeting a gnomically integrated fluorescent reporter gene. In addition, this toolset will be used to increase 2-pyrone 4,6-dicarboxylic acid (PDC), β-ketadipate (βKA), and muconate titers by temporally repressing downstream enzymes that funnel these desired products into central metabolism.

To optimize CRISPR-Cas gene editing, the transformation protocol from Sun et al. 2018 was improved to increase the colony forming units by 100-fold while keeping the editing efficiency

at 100%. The minimum homology arm (HA) length requirements for gene deletion was determined and the HA length requirements for gene integration and introducing single codon mutations are underway.

Together these tool optimization studies will enable high-throughput and multiplexed gene editing and gene silencing experiments to be conducted in *P. putida*. This will be leveraged to rapidly map genotype-phenotype relationships as well as engineer high-titer strains.

References

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