Engineering CRISPR-Cas Systems for Genome Editing in *Pseudomonas putida* KT2440 and *Clostridium thermocellum*

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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 addresses 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, C6 esters and hydrocarbons) using CBP at high rates, titers and yield in combination with cotreatment, pretreatment or catalytic upgrading. CBI will maximize product value by *in planta* modifications and biological funneling of lignin to value-added chemicals.

Efficient microbial conversion of lignocellulose to fuels and chemicals is key to an economically viable bioproduction platform, particularly if coupled with feedstocks designed for optimal microbial performance. At their core, current approaches for accelerated domestication of model organisms such as *E. coli* mirror the cycle of "Design, Build, Test," revealing underlying design principles that inspire creative solutions to modern engineering challenges. The programmability of CRISPR-Cas machinery enables library based high-throughput and multiplexed experiments that have allowed for rapid genotype-phenotype mapping (1), protein engineering (2), strain engineering (3), and gene discovery (4). Our goal is to develop efficient CRISPR-mediated genome editing systems to enable these and other cutting-edge genome editing technologies in CBI microbes and feedstocks.

Both CRISPR-Cas editing and interference using the *Streptomyces pyogenes* Cas9 system have been demonstrated in the bacterium *Pseudomonas putida* KT2440, a promising candidate for the industrial production of renewable chemicals from lignin. CRISPR-interference (CRISPRi) was optimized for *P. putida* 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. In addition, we have quantified the dynamic range of repression by targeting a genomically-integrated fluorescent reporter as well as key metabolic genes that compete with pathways to target products to increase titers. To optimize CRISPR-Cas gene editing, the transformation protocol from Sun et al. 2018 (5) 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 and introduction of single codon mutations were determined. We are utilizing this system to recapitulate mutants from adaptive laboratory evolution (ALE) experiments and generate target mutagenesis libraries to evaluate protein function. In addition, we are working with JGI to generate a genome wide guide RNA library (237,000 guides) to identify functional guides for gene editing and repression via CRISPRi to

expand this system for genome scale studies.

Although the *Streptomyces pyogenes* Cas9 system has been utilized across a number of microbial and eukaryotic platforms, unfortunately it is not active in the growth temperature range of *Clostridium thermocellum* (55-60ºC), a thermophilic bacterium capable of directly converting cellulose to sugars, bypassing the need for chemical processing of lignocellulosic feedstocks. To enable CRISPR editing, we adapted and validated both the Type I-B native CRISPR system in *Clostridium thermocellum,* as well as the Cas9 system from *Geobacillus stearothermophilus* (6,7). Multiple attempts for CRISPR/Cas homology directed genome repair were largely unsuccessful for both systems, most likely due to the low efficiency of homologous recombination in *C. thermocellum.* To overcome this limitation, recombineering machinery was isolated from the thermophilic organism *Acidithiobacillus caldus* and expressed in *C. thermocellum*. An increase in homologous directed repair was observed in *C. thermocellum* strains expressing recombineering machinery when compared to parental strains, enabling a more rapid and efficient genetic engineering system in *C. thermocellum*. We are currently working on the evaluation of promoter systems to enable inducible CRISPRi in *C. thermocellum.*

Ultimately, we aim to utilize these CRISPR/Cas systems for rapid, HTP methods for phenotype-to-genotype discovery in both *P. putida* and *C. thermocellum* such as: 1) rational protein engineering, 2) complete residue substitution libraries, 3) pathway optimization, and 4) discovery of new gene functions by genome-wide targeting strategies. These tools will expand and accelerate the canonical the "Design, Build, Test" cycle in support of CBI research needs.

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