**Engineering CRISPR/Cas Systems for Genome Editing in *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 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.**

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. Although understanding of complex biological systems and "design rules" for metabolic engineering of model organisms have progressed significantly, we are still unable to effectively engineer many desired properties necessary for a sustainable and efficient bioprocessing platform in non-model microbes and plant feedstocks. Therefore, it is essential that we expand genetic engineering capabilities in novel non-model systems with desired traits for bioprocessing to accelerate advancement of the U.S. bioeconomy.

Advances in DNA synthesis and powerful genome editing tools such as CRISPR (**C**lustered **R**egularly-**I**nterspaced **S**hort **P**alindromic **R**epeats) have led to a rapid expansion of genome engineering in model and now increasingly in non-model organisms. 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. 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* (55-60$^{o}$C), a thermophilic bacterium capable of directly converting cellulose to sugars, bypassing the need for chemical processing of lignocellulosic feedstocks. Diverse CRISPR systems exist amongst Archaea and Bacteria, and the genome of *C. thermocellum* DSM1313 encodes two Type III and one Type I CRISPR/Cas systems. Unlike Type II systems (e.g. Cas9), which require only a single Cas protein for targeting (via the guide RNA, or gRNA), Type I and III systems require a multi-subunit Cas complex. Since Type III systems generally target RNA instead of DNA, we focused on characterizing the native Type I system as a tool for genome editing in *C. thermocellum.* We have been exploring the Type I-B CRISPR system in *Clostridium thermocellum* with the goal of using it for gene deletion and editing. In order to overcome sensitivity issues associated with modest expression of the endogenous CRISPR locus genes and spacers in *C. thermocellum,* we constructed an artificial CRISPR spacer expression cassette to probe CRISPR activity. We used this construct in a plasmid

transformation inhibition assay to test and validate predicted PAM sequences. This assay coupled with a PAM library depletion assay allowed us to identify the optimal PAM sequence of 5'-T (preferred, or C)/N/A, G or T-3' in *C. thermocellum*. In efforts to further enhance/optimize CRISPR activity in *C. thermocellum*, we inserted heterologous promoters upstream of the Cas operon. Quantitative PCR analysis showed upregulation to various degrees of the operon in all strains relative to the parental strain LL1299. This upregulation correlated with an increase in CRISPR activity as assayed in the plasmid transformation inhibition assay. Thus far efforts at gene editing have focused on the *pyrF* locus. We show a spacer dependent decrease in transformation, likely due to targeting of the wildtype *pyrF* locus. As expected, this decrease in transformation is higher in the promoter engineered strains where the Cas operon is expressed at elevated levels relative to those in LL1299. As seen in other *Clostridium* strains, we find that serial transfer of individual transformants increases the appearance of the pyrF deletion. allele. Current efforts are aimed at increasing the efficiency of CRISPR editing.

In addition, three thermophilic Cas9 systems have recently been characterized (1–3) and were additionally evaluated for activity/genome editing in *C. thermocellum*. A toxicity assay indicated that the *Geobacillus stearothermophilus* thermophilic Cas9 was the most active in *C. thermocellum.* However, multiple attempts for CRISPR/Cas9 homology directed genome repair were unsuccessful most likely due to unregulated expression of Cas9 as well as the low efficiency of homologous recombination in *C. thermocellum.* To overcome the first limitation, we placed Cas9 expression under a tightly regulated inducible promoter. To overcome the second 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. We are now poised to enable rapid CRISPR/Cas9 genetic engineering in *C. thermocellum*.

We expect that CRISPR systems can be harnessed to not only accelerate precise genome editing in CBI organisms, but also to create targeted, *trans*-acting regulatory systems as has been demonstrated in other microbes including CRISPR interference (CRISPRi) for targeted knockdown of genes of interest to begin to determine gene-to-trait attributes towards desired phenotypes. Preliminary data on these evaluations will be presented.

Ultimately, we aim to utilize these CRISPR/Cas systems for a rapid, HTP method for phenotype-to-genotype discovery in *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 for gene-to-trait discovery in support of CBI research needs.

**References**


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