Identification and Optimization of CRISPR Systems for Genome Editing in *Pseudomonas putida* and *Clostridium thermocellum*

Jacob Fenster[1], Audrey Watson[1], Julie Walker[1], Emily Freed[1], Sandra Notonier[2], George Peabody[3], Anthony Lanahan[4], Gregg Beckham[2], Adam Guss[3], Dan Olson[4], Jeff Cameron[1], Ryan Gill[1], Lee Lynd[4], Carrie Eckert[1,2*] (carrie.eckert@nrel.gov), and **Gerald A. Tuskan**[3]

[1]Renewable and Sustainable Energy Institute, University of Colorado, Boulder CO; [2]National Renewable Energy Laboratory, Golden, CO; [3]Oak Ridge National Laboratory, Oak Ridge, TN; [4]Dartmouth College, Hanover, NH.

http://cbi.ornl.gov

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. And 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. 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 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. To enable these and other cutting-edge genome editing technologies in CBI microbes, our first goal is to develop efficient CRISPR-mediated genome editing systems in both *Pseudomonas putida* and *Clostridium thermocellum*.

Genome editing utilizing Cas9 from *Streptococcus pyogenes* (a Type II CRISPR/Cas system) has recently been demonstrated in *P. putida* KT2440 (1). The editing efficiency is low, so we are also investigating a number of strategies to improve CRISPR-based editing and recombination efficiency.

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 *C. thermocellum* (55-60^{o}C). 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 are characterizing the native Type I system as a tool for genome editing in *C. thermocellum.* In addition, two thermophilic Cas9 systems have recently been characterized (2,3) and are currently being evaluated for genome editing in *C. thermocellum*.

We expect that CRISPR systems can be harnessed to not only accelerate precise genome editing in CBI microbes, but also to create targeted, *trans*-acting regulatory systems as has been demonstrated in *E. coli* and cyanobacteria (4,5). Therefore, we are currently evaluating a CRISPR interference (CRISPRi) system in *P. putida* to allow 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 *P. putida* and *C. thermocellum*. **CRI**SPR **E**n**A**bled **T**rackable Genome **E**ngineering (CREATE) couples multiplexed, high efficiency CRISPR-based genome editing with massively parallel oligomer synthesis. This method enables generation of hundreds of thousands of designer modifications (via pooled oligo microarrays) and, by means of a trans-acting barcode, the simultaneous sequence-to-activity mapping of all of such modifications (6). In conjunction with a selectable and/or screenable phenotype (e.g., improved growth, substrate tolerance, fluorescence, etc.), this technique can be applied to determine genotype-to-phenotype relationships for: 1) rational protein engineering, 2) complete residue substitution libraries, 3) pathway optimization and 4) discovery of new gene functions by genome-wide targeting strategies. This method will expand and accelerate the canonical the “Design, Build, Test” cycle for gene-to-trait discovery in support of CBI research needs.

References

1. Aparicio T, de Lorenzo V, Martínez-García E. CRISPR/Cas9-Based Counterselection Boosts Recombineering Efficiency in Pseudomonas putida. Biotechnol J. 2017 Oct 23
2. Mougiakos I, Mohanraju P, Bosma EF, Vrouwe V, Finger Bou M, Naduthodi MIS, et al. Characterizing a thermostable Cas9 for bacterial genome editing and silencing. BioRxiv. 2017 Aug 18.
3. Harrington L, Paez-Espino D, Chen JS, Ma E, Staahl BT, Kyrpides NC, et al. A thermostable Cas9 with increased lifetime in human plasma. BioRxiv. 2017 May 16.
4. Luo ML, Leenay RT, Beisel CL. Current and future prospects for CRISPR-based tools in bacteria. Biotechnol Bioeng. 2016 113(5):930–943.
5. Gordon GC, Korosh TC, Cameron JC, Markley AL, Begemann MB, Pfleger BF. CRISPR interference as a titratable, trans-acting regulatory tool for metabolic engineering in the cyanobacterium Synechococcus sp. strain PCC 7002. Metab Eng. 2016 38:170–179.
6. Garst AD, Bassalo MC, Pines G, Lynch SA, Halweg-Edwards AL, Liu R, et al. Genome-wide mapping of mutations at single-nucleotide resolution for protein, metabolic and genome engineering. Nat Biotechnol. 2017 35(1):48–55.

The Center for Bioenergy Innovation is a U.S. Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science.