**Design and engineering of native regulatory networks in non-model microbes**

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**Project Goals: Our objective is to develop a new standard for the engineering of microbial systems based on rational design, engineering, and optimization of hybrid regulatory networks. We envision a future biorefinery that is based on the development of designer organisms that have exquisite and predictable control architectures governing the expression of a range of valuable traits. Computer aided design platforms will guide the assembly of synthetic constructs containing orthogonal heterologous circuits to recode native regulatory networks. Together, these will enable predictable and dynamic control of multiple designer phenotypes such as: i) growth on various feedstocks in consolidated bioprocesses, ii) feedback control to mitigate accumulation of toxic metabolites, iii) production of target molecules (C3-C4 alcohols), and/or iv) robustness to process upsets (e.g. temp., phage). The focus of this proposal is to develop the technical and computational infrastructure to enable this vision. We will develop this platform first in the model organism *E. coli* and then in DOE relevant non-model organisms.**

The realization of a sustainable bioeconomy requires our ability to understand and engineer complex design principles for the development of platform organisms capable of efficient conversion of cheap and sustainable feedstocks (e.g. sunlight, $CO_2$, non-food biomass) to biofuels and bioproducts at sufficient titers and costs. Despite recent advances in DNA synthesis allowing for the construction of small, synthetic genomes and the development of high-throughput genome editing and metabolic engineering tools in model microbes, our ability to design genomes and predict design principles for intricate functions such as photosynthesis and tolerance are still limiting. In the proposed work, we will leverage our knowledge and expertise in these cutting-edge synthetic biology techniques currently only available for model microbes by partnering with expert collaborators in the adaptation of these methods for DOE-relevant microbial systems, accelerating and expanding genome editing capabilities for metabolic engineering in these organisms. Through doing so, we will be able to more quickly uncover genotype to phenotype relationships to better engineer these microbes for optimal production of chemicals and fuels from renewable feedstocks.

*Synechococcus* sp. PCC7002 is attractive as a photosynthetic chassis for sustainable biochemical production because of its fast growth rate and its ability to grow using only sunlight as an energy source to convert $CO_2$ into products. Transcriptional responses to different growth conditions have been measured, and the key players in RNA-turnover, a major process controlling cellular production, have been characterized by collaborator Jeff Cameron. In addition, a mass transport and kinetic modeling of the PCC7002 $CO_2$ concentrating mechanism identified potential ways to increase photosynthetic growth in industrial conditions, and metabolic flux models have been developed for PCC7002 to identify changes predicted to increase biofuel production. PCC7002 is naturally competent for DNA uptake and targeted gene deletions can be routinely performed at relatively high efficiencies using native homologous recombination machinery. A robust synthetic biology toolkit is available including promoter and ribosome binding site libraries, orthogonal/inducible promoter systems, counter-selectable markers, multiple neutral integration

sites in the genome, and an inducible CRISPR-interference system to enable titratable repression of endogenous genes.

*Zymomonas mobilis ZM4* is a facultative anaerobe that is of interest for the fermentation of biomass sugars to biofuels and bioproducts, especially those branching from pyruvate. Collaborator Min Zhang demonstrated that carbon flux can be deviated from ethanol production at the pyruvate node into 2,3-butanediol as well as to farnescene (unpublished). They have generated a large volume of –omics data, providing insights into hydrolysate tolerance and feedstock utilization. Transformation efficiencies with a variety of vectors have been optimized, homologous recombination is performed routinely for genome engineering, and a number of functional promoters, both constitutive and inducible, have been characterized. In addition, heterologous expression of Cas9 and targeting gRNA was demonstrated to cure native plasmids in *Z. mobilis* (Cao et. al., 2017), representing initial proof of concept for CRISPR-based gene editing and regulation.

The yeast *Kluveromyces marxianus* is an interesting biomass sugar utilizing organism because of its tolerance to low pH, higher temperatures, and high flux to pyruvate. Numerous tools have been developed to enable directed engineering of *K. marxianus* by collaborator Ian Wheeldon and others. Centromeric episomal plasmid systems have been well established for this organism, and CRISPR-Cas9 has been developed for genome editing and gene knockdown (via CRISPR interference) technologies have been developed to enable rapid genome-wide screening efforts.

We are currently working to adapt genome engineering tools for each of the above target microbes to enable technologies such as CRISPR interference (CRISPRi) for gene knockdown and CRISPR-Enabled Trackable Geneome Engineering (CREATE) for multiplexed editing. The investigators on this project have all developed an array of vectors for Cas9/dCas9 and gRNA expression in a range of organisms, and a large number of additional vectors are available through Addgene (http://www.addgene.org/). These vectors will be adapted, as necessary, for stable replication and predictable expression in each of the testbed organisms. Targeting, cutting, and recombination efficiencies will be evaluated and optimized for Cas9/gRNA utilizing an appropriate screen/selection method (antibiotic resistance, auxotrophy, counterselective, colorimetric, etc.). In addition, we are evaluating and optimizing knockdown efficiencies using dCas9 in a similar fashion.

Once CRISPR-based genome editing is validated in each of the non-model systems, we will adapt the CREATE method for targeting global regulators in the testbed strains to mirror the regulator libraries utilized by other teams on this project (Ryan Gill and Chris Voigt) to expand the search space for regulatory control switches. Utilizing existing pathway maps for each organism, we will build pathway prediction models for each to identify global regulators to target. Information gleaned from the selection experiments (improved growth, tolerance, etc.) will guide further improvements to the models for development of synthetic regulatory networks by another team member (Adam Arkin). Together, this work will provide a blueprint for the development of systems to accelerate the engineering of non-model microbial systems as bioproduction chassis organisms.

**References**

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*This research is supported by the Office of Biological and Environmental Research in the DOE office of Science, grant DE-SC0018368.*