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 will create both molecular tools and computational infrastructure to meet this overall goal in both *E.coli* and DOE relevant non-model organisms. Through increasing our understanding of native regulatory networks and then using heterologous synthetic circuits to recode them, we 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). Here we focus on expanding our work into non-model microbes to engineer designer organisms that have exquisite and predictable control architectures governing the expression of a range of valuable traits.

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 tolerance are still limiting. In the proposed work, we will leverage our knowledge and expertise in 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.

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 farnesene (Yang et. al. 2016). 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.

We are currently working to adapt genome engineering tools for *Zymomonas* 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 developed an array of vectors for Cas9/dCas9/Cas12a 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 have been adapted, as necessary, for stable replication and predictable expression. Targeting, cutting, and recombination efficiencies are being evaluated and optimized for Cas9/Cas12a/gRNA utilizing an appropriate screen/selection method (antibiotic resistance, auxotrophy, counterselective, colorimetric, etc.). We will evaluate and optimize knockdown efficiencies using dCas9 in a similar fashion. Although we found that Cas9 expression was toxic in *Z. mobilis,*we have achieved high editing efficiency with the Cpf1/Cas12a system. We are additionally working on developing a dCas9 system for CRISPRi.

Once CRISPR-based genome editing is validated, we will adapt the CREATE method for targeting global regulators 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 the organism, we will build pathway prediction models 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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