**Engineering of Regulatory Networks for Complex Phenotypes in *E. coli* and *S. cerevisiae***

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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 organisms *E. coli* and *S. cerevisiae* and then in DOE relevant non-model organisms.**

The sustainable production of biofuels and bioproducts is of continued importance in light of increasing concerns about climate change and energy security. Advances in metabolic engineering, synthetic biology, and systems biology have provided a number of strategies for the more rapid design, construction, and testing of model systems for the production of next generation industrial compounds. However, the titer and productivity of engineered strains are still below those required for economic production. The rate limiting step is no longer our ability to construct designer strains, but rather how to design and engineer increasingly complex networks of combinatorial phenotypes required for the economic and sustainable production of these biofuels and other bioproducts. The core challenges are: i) the general lack of mechanistic understanding required to predictably rewire targeted phenotypes; and ii) the size of the combinatorial mutational space spanning complex phenotypes is much larger than the size that can be searched on laboratory timescales.

To address these issues, we developed a foundation for forward engineering of regulatory control architectures, which combines CRISPR Enabled Trackable Genome Engineering (CREATE; developed in the Gill lab) and forward engineering of *E. coli* and *S. cerevisiae* regulatory networks to access complex targeted phenotypes. We designed, constructed, and mapped libraries of more than 100 regulatory genes containing more than 100,000 specific mutations to perturb the *E. coli* regulatory network. We performed growth competition experiments for library mutants conferring increased tolerance to a variety of industrially-relevant compounds including

furfural, styrene, acetate, isopropanol, and isobutanol. We additionally identified *E. coli* regulatory gene mutants that had increased styrene or isobutanol production. We performed in-depth analyses of the mutations conferring increased tolerance and/or production phenotypes to gain a better understanding of global regulation in *E. coli*.

We additionally mapped over 83,000 mutations in 47 regulatory proteins in *S. cerevisiae* and used these data to identify mechanisms conferring tolerance to isopropanol and isobutanol in yeast. Future work, done in collaboration with the Cell Architecture Lab at the Novo Nordisk Foundation Center for Biosustainability, will focus on designing novel strategies to engineer regulatory networks in *S. cerevisiae* by investigating post-translational modification signaling, such as phosphorylation, ubiquitylation, and acetylation. Post-translational modifications provide a rapid and reversible method to regulate protein functions; therefore, they are an attractive target for modifying protein activities. We additionally aim to build designer libraries to replace endogenous promoters with synthetic promoters to investigate the functional impact of gene dose on organismal traits. Yeast CREATE libraries will be evaluated for industrially relevant stress resistance under model bioreactor conditions.

*This research is supported by the Office of Biological and Environmental Research in the DOE Office of Science under contract number DE-SC0018368.*