Engineering of Regulatory Networks for Improved C3-C4 Alcohol Tolerance and Production 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 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 fuel-grade compounds (e.g. C3–C5 alcohols). However, the titer and productivity of engineered strains that produce C3-C5 alcohols 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*/yeast 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* and yeast global regulatory networks. We performed growth competition experiments for library mutants

conferring increased C3-C4 alcohol tolerance and also screened for increased alcohol production. The titer of isopropanol and isobutanol in the best producing *E. coli* mutants were improved 3-fold and 2-fold compared to the parent strain, and the tolerance for isopropanol and isobutanol were increased to 30 g/L and 10 g/L, respectively. The tolerant yeast mutants tolerated 60 g/L isopropanol and 15 g/L isobutanol while the parent strain did not grow in these high concentrations of alcohol.

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