Functional genetic screening in the thermotolerant yeast *Kluyveromyces marxianus*

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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. While substantial research has been put toward advancing model hosts such as *Saccharomyces cerevisiae* for chemical biosynthesis, less effort has been made with non-conventional hosts. In this context, we seek to create a functional CRISPR-based genomic screening tool to explore the genetics and metabolism of the yeast *Kluyveromyces marxianus*. This yeast is targeted for this project because it is thermotolerant and natively possesses the ability to uptake and metabolize a range of C5, C6, and C12 sugars. The outcomes of this work will not only help advance our understanding of *K. marxianus* and enable the engineering of new strains but may also lead to new genome editing technologies and improved our fundamental understanding of other non-model organisms.

The key element to enable the utilization of the low pH yeast *K. marxianus* in industrial applications is the development of improved genetic tools to more quickly and effectively manipulate the genome for genotype-phenotype discovery and metabolic engineering. There are efforts to develop these tools in *K. marxianus*, mainly aiming at the modification of metabolic pathways. *K. marxianus* has advantages over other yeast species, mainly because it has greater thermal stability (>40^{o}C), low pH tolerance, a relatively fast growth rate, and a highly resistant cell membrane. Fermentative processes, when carried out at higher temperatures, reduce cooling costs in addition to reducing problems caused by contamination. *K. marxianus* also has a high secretory capacity in relation to *S. cerevisiae*, due to properties such as appropriate glycosylation and strong signal peptides. This strain has been viewed as an alternative to *S. cerevisiae* in 2nd generation ethanol processes as it is able to naturally assimilate a variety of sugars in addition to glucose, such as pentose, hexose, arabinose, cellobiose, lactose and xylose, as well as some toxic compounds present in some sources of lignocellulosic biomass.

In collaboration with JGI's DNA Synthesis team and Ian Wheeldon at UC-Riverside, a genome-wide CRISPR-Cas9 library has been created. This new tool will then be used to define essential genes that support growth on a range of substrates as well as to identify genes essential to high temperature and low pH growth. Library validation and growth screen experiments will require next-generation sequencing amounting to 10 sets. Essential genes necessary for growth on glucose, xylose, glycerol, and lactose at high temperatures (37^{o}- 50^{o}C) will be identified. In addition, other conditions as tests under low pH, such as 2.5, 3 and 3.5, and tolerance to isobutanol will be performed. These screening experiments will require up to 8 different experiments each requiring up to 400 million next-generation sequencing reads. The outcomes of this work will not only help advance our understanding of *K. marxianus* and enable the engineering of new strains, but may also lead to new tools, technologies, and fundamental understanding of other non-model organisms.

This research is supported by the Office of Biological and Environmental Research in the DOE Office of Science.