Functional genomics of lipid accumulation in the oleaginous yeast *Rhodosporidium toruloides* using randomly bar-coded TDNA-Seq

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Project Goals: Conduct large-scale mapping of genotype to phenotype in oleaginous yeast, focused on the genes underlying lipid production, plant feedstock hydrolysate tolerance, low-oxygen metabolism, and co-utilization of sugars in plant material. The yeast chosen for this study, *Rhodosporidium toruloides,* has several advantages over more traditional model yeasts, including its native ability to metabolize the sugars in plant hydrolysates (i.e., glucose, xylose, arabinose, and cellobiose) and high *de novo* lipid productivity. Techniques for *R. toruloides* genetics and genome engineering will be developed and used to map the determinants of complex growth and lipid productivity traits in wild isolates and engineered strains. Establishment of a robust, versatile model yeast that natively accumulates high lipid levels will enable greater flexibility in developing new biofuels that produce industrial strains and will provide fundamental insights into the origins of complex traits useful for biofuels production.

The ability to rapidly functionally annotate genomes of non-model fungi is a barrier to developing and exploiting new host organisms for biotechnology and studying interesting fungal traits. We study the oleaginous basidiomycetous yeast *Rhodosporidium toruloides*, which can accumulate up to ~70% of its dry cell weight, primarily as triglycerides (TAG). These triglycerides can be extracted and converted into biodiesel or the high acetyl-CoA flux required for lipid accumulation could be harnessed for the production of fatty acid derived biofuels/chemicals. Furthermore, *R. toruloides* can also generate relatively high levels of carotenoid compounds, consume a wide array of carbon sources and tolerate common sources of inhibition found in biomass waste substrates. However, little is known about the *R. toruloides* genetics of these useful traits. Here, we have developed a functional genomics technology that is an extension of RB-TnSeq, called RB-TDNA-Seq that can be used in fungal systems. Instead of transposon mutagenesis, our method exploits the efficiency of *Agrobacterium tumefaciens* mediated transformation (ATMT) to randomly integrate a transfer DNA (TDNA) fragment into the fungal host genome. ATMT mutagenesis has been reported in hundreds of fungal species; therefore, our method should be widely applicable. We have performed proof of concept experiments demonstrating that we can quantify mutant strain fitness using a growth-based assay, and also have developed an assay based on buoyant density to identify genes required for lipid accumulation. To demonstrate our fitness-based assay, we used a ~50,000 member mutant pool to identify genes required for amino acid biosynthesis. By comparing the fitness profile in

minimal media with synthetic complete media, or media supplemented with single amino acids, we identified arginine, methionine, proline, and leucine auxotrophs. In another pilot experiment using a smaller mutant pool (~6,000 strains) we successfully segregated strains with altered lipid accumulation by differences in their buoyant density. Lipid production was induced by growth in nitrogen-limited conditions, and then cultures were segregated into high-density, medium-density, or low-density fraction by progressive centrifugation in increasing concentrations of sucrose. Over 900 mutant strains representing disruptions in over 700 genes were significantly enriched in the high-density or low-density fractions as compared to the input culture. These genes represented a wide range of cellular and biochemical functions including lipid metabolism, nutrient sensing, the cellular endomembrane system, transporters and many uncharacterized proteins. Ten genes with multiple insertions that had consistent enrichment patterns, or for which a single mutant strain was strongly enriched, were then deleted by homologous recombination using a drug resistance marker in a Ku70 deletion strain background. Preliminary experiments comparing TAG accumulation in these strains (as measured by BODIPY 495/503 intensity) to the Ku70 background confirmed that four strains had unambiguously altered lipid accumulation, three modest but significant lipid phenotypes, and three had unaltered lipid accumulation or phenotypes too subtle to measure with this protocol. Among the genes with strong lipid phenotypes were homologs to the human genes seipin and tuberin, shown to be involved in lipid metabolism in several other model systems. We have now scaled up our ATMT mutagenesis protocol and generated a randomly integrated, bar-coded mutant pool of *R. toruloides* consisting of ~400,000 members using this technology. This large mutant pool and the methods we developed will now enable us to perform genome-wide fitness and lipid accumulation screens.

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