**Engineering Anaerobic Gut Fungi for Lignocellulose Breakdown**

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**Project Goals: The goal of this project is to engineer anaerobic gut fungi as novel platform organisms for biofuel production from plant material. To accomplish this goal, a panel of anaerobic fungi will be isolated from different herbivores and screened for their ability to degrade lignocellulose. The basic metabolic networks that govern lignocellulose hydrolysis within anaerobic fungi will also be determined, and models will be generated to describe how important enzyme groups are coordinated during breakdown. Using this information, genetic transformation strategies to manipulate gut fungi will be developed, which would endow them with enhanced functionality against a range of industrially relevant substrates. Collectively, this information will establish the molecular framework for anaerobic fungal hydrolysis, and will guide in the development of lignocellulosic biofuels.**

Anaerobic fungi are the primary colonizers of biomass within the digestive tract of large herbivores, where they have evolved unique abilities to break down lignin-rich cellulosic biomass through invasive, filamentous growth and the secretion of powerful lignocellulolytic enzymes and enzyme complexes (cellulosomes). Despite these attractive abilities, considerably less genomic and metabolic data exists for gut fungi compared to well-studied anaerobic bacteria and aerobic fungi that hydrolyze cellulose. This presents a significant knowledge gap in understanding gut fungal function, substrate utilization, and metabolic flux, which has prohibited the genetic and functional modification of gut fungi. Our approach combines next-generation sequencing with physiological characterization to establish the critical knowledge base to understand lignocellulose breakdown by gut fungi.

We worked with collaborators at the Joint Genome Institute (JGI) to sequence genomes of the anaerobic fungi *Neocallimastix californiae*, *Anaeromyces robustus*, and *Piromyces finnis*, which were isolated from large herbivores. Our recent publication in Nature Microbiology released the first high-quality genomes of the anaerobic gut fungi (results available on Mycocosm). With the advent of PacBio sequencing, long reads averaging 10,000bp in length were sufficient to remedy the assembly of highly repeat-rich regions of the genomes that could not be resolved with Illumina-based approaches. These results also enabled further epigenetic analysis of the fungal genomes (ongoing collaboration with Igor Grigoriev's group at JGI), that highlighted regions of the genomes that are highly transcribed (Mondo, et al, Nature Genetics).

In previous years of this project, we established that gut fungi secrete a large number of cellulolytic enzymes, which form cellulosomes that may be physically attached to the cell. However, the enzyme components, modular assembly mechanism, and functional role of fungal cellulosomes during biomass breakdown remained unknown. Although the basic interaction of these complexes is a modular cohesion-dockerin binding similar to that observed in bacteria, the fungal dockerin and scaffoldin domains have no similarity to their bacterial counterpart. Previously obtained genomic and transcriptomic data for three fungal strains isolated by our lab enabled us to identify a "parts list" for fungal cellulosomes, including a set of novel scaffolding proteins that biochemically interact with dockerin-fused enzymes from fungi (Haitjema, 2017 *Nature Microbiology*). Further, our previous work has revealed that (i) only some of the scaffoldin proteins have putative transmembrane helix domains, (ii) many of the dockerin domain proteins (DDPs) do not identify as carbohydrate active enzymes (CAZy) and (iii) fungi still secrete up to 50% of their CAZymes as free enzymes (lacking a dockerin domain) (Haitjema, 2017 *Nature Microbiology*). Finally, comparative genomics against anaerobic bacteria and other strains of anaerobic fungi revealed that a number of CAZyme domains identified in the fungal genomes originated from bacteria. This finding indicates that fungi and bacteria that co-exist in the rumen of large herbivores may have exchanged genetic information, allowing for the optimization of biomass degradation in these anaerobic systems. This finding is surprising, as horizontal gene transfer in this respect is typically observed to occur within life forms that share the same kingdom of life – it is seldom described to occur from prokaryotes to eukaryotes.

Building from these insights, we developed a biphasic fermentation scheme that combines the lignocellulolytic action of anaerobic fungi with domesticated microbes for bioproduction. When grown in batch culture, anaerobic fungi release excess sugars from both cellulose and crude biomass due to a wealth of highly expressed carbohydrate active enzymes (CAZymes), converting as much as 49% of cellulose to free glucose. This sugar-rich hydrolysate readily supports growth of *S. cerevisiae*, which can be engineered to produce a range of value-added chemicals. Further, reconstruction of metabolic pathways from transcriptomic data reveals that anaerobic fungi do not catabolize all sugars that their enzymes hydrolyze from biomass, leaving other carbohydrates such as galactose, arabinose, and mannose available as nutritional links to other microbes in their consortium. Overall, these results suggest that anaerobic fungi provide a nutritional benefit to the rumen microbiome, which can be harnessed to design synthetic microbial communities that compartmentalize biomass degradation and bioproduct formation.

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