

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.

At the outset of this project, a fungal enrichment experiment was conducted to isolate biomass-degrading fungi from large herbivores at the Santa Barbara Zoo that would serve as model systems. These isolates have officially been assigned species names, and are indexed in the Index Fungorum (<http://www.indexfungorum.org>). In the past 2 years, our group has worked with collaborators at the Broad Institute of MIT and Harvard, as well as the Joint Genome Institute (JGI) (via approved CSP projects) to sequence transcriptomes and genomes of the anaerobic fungi *Neocallimastix californiae*, *Anaeromyces robustus*, and *Piromyces finnis*. Through this work, we found that the number of biomass-degrading genes in the anaerobic fungi was richer than any other microbe yet described in nature. Compared to “higher” (e.g.

more evolved fungi), the more primitive anaerobic fungi are equipped with a rather even distribution of cellulose-degrading enzymes, xylan-degrading enzymes, and accessory enzymes that have been largely lost to evolution. This illustrates not only their powerful biomass-degrading abilities, but also the potential to identify completely novel sequences in their genomes that could be exploited for biotechnology. More recently, we have worked with the JGI to release the first high-quality genomes of the anaerobic gut fungi (manuscript in progress, results available on MycoCosm).

Compared to model organisms, there is a relative dearth of information about the metabolism of anaerobic gut fungi. To address this issue, we used transcriptomic information to identify enzymes from critical metabolic processes for the first time. Transcripts identified in the *de novo* assembled transcriptomes were functionally annotated using a combination of NCBI BLAST, EMBL-EBI InterProScan, and Gene Ontology mapping. Enzymes were identified by their Enzyme Commission (EC) numbers, which were only assigned to transcripts with a high similarity to InterPro protein domains. Combining this information with metabolic maps from KEGG databases, the core metabolic maps were filled in and the sugars that could be metabolized by the fungus were identified. This analysis revealed that the fungus contains all of the enzymes required for glycolysis through ethanol fermentation, but takes an irreversible route as it is missing phosphoglycerate mutase (EC:5.4.2.11), which indicates that the gut fungi cannot perform gluconeogenesis. Furthermore, all enzymes necessary for the metabolism of fructose, mannose, sucrose, α -galactose, and xylose were present.

Finally, we have taken the first major steps towards a method to stably genetically transform anaerobic gut fungi. Beyond the obvious lack of knowledge of their genomes, the very thick cell wall of anaerobic fungi (greater than 5 μ m) often prevents delivery of any amount of DNA. So far, we have shown that gut fungal zoospores (cells in the early stage of their life cycle) are amenable to transformation by electroporation (DNA uptake during electroshock). This optimized procedure was the result of a design of experiments approach, where electrotransformation, biolistic transformation, and chemical transformation methods were evaluated as applied to fungal zoospores and mature zoosporangium. In our experiments, only during electroporation are zoospores capable of taking in foreign extracellular plasmid DNA, and then are able to express the genetic reporter encoded on the plasmid as they mature. In this case, the genetic reporter is the GusA enzyme, which hydrolyzes the reagent X-gluc, creating an easily observable blue colony formation. Future experiments will leverage this transformation breakthrough to genomically modify the model fungal systems for the first time.

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