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FINAL TECHNICAL REPORT

"Tracking Down Cheaters: Molecular Analysis of Carbon Consumption by Organisms That Do Not Contribute to Extracellular Enzyme Pools"

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The overriding objective of our work is to integrate physiological and community ecology of belowground organisms into understanding of soil carbon dynamics to improve predictions of terrestrial ecosystem models. This includes using metagenomics and metatranscriptomics-based methods to understand microbial interactions affecting decomposition and soil carbon dynamics. The focus of the majority of the work directly related to this project was on "cheating", a poorly understood microbial interaction with a potentially large effect on decomposition. Progress on projects related to this topic are described below first. In addition, we study plant and microbial traits and plant-microbe interactions that affect species distributions and soil carbon, and also develop bioinformatics tools to increase the power of ecological inferences that can be obtained from omics-based sequence data. These additional accomplishments are described at the end of the report if they have reached publication.

Background on "cheating" in decomposition

Currently, biogeochemical models of decomposition that underlie ecosystem and global climate models are based on simple first-order decay kinetics without consideration of the microbiological basis of the process. However, there is a critical need to incorporate microbial physiological and community dynamics into these models to increase our ability to predict decomposition under non-equilibrium conditions, such as due to short-term perturbations or long-term changing climate. Most carbon in plant litter is locked in a mixture of complex polymers that make up the plant cell wall. Decomposition of these polymers is performed biochemically by extracellular enzymes (ECE) secreted by decomposer microorganisms that degrade polymers into simple molecules, which can then be taken up directly by microbial cells. We refer to the microbes secreting ECE as ***"investors"*** because of the large carbon and nutrient cost associated with production of ECE, and because of the expected return of dissolved labile substrate. ***"Cheating"*** during decomposition involves ***consumption of carbon by microorganisms that do not contribute to pools of extracellular enzymes (ECE)***. Cheating is likely a widespread feature of decomposer communities, but is poorly understood and not included in models of decomposition.

Our approach to determine which microorganisms were cheating and how much carbon they consumed involved using stable isotope probing of genomic DNA coupled with metatranscriptomics. Obligate cheaters, opportunistic cheaters, and investors will all consume ^{13}C-labeled monomers, and their gene sequences will be present in ^{13}C-labeled metagenomes. Opportunistic cheaters have genes for ECE but are not expressing them; hence their ECE genes would not be present in the metatranscriptome. Obligate cheaters lack genes for ECE, which will be used to distinguish them from opportunistic cheaters and investors. Much of our activity has been focused on developing tools to make these distinctions, beginning with model organisms with genome sequences available.

1. Physiological and transcriptomic responses in *Talaromyces stipitatus* and *Schizophyllum commune* to substrate fluctuation.

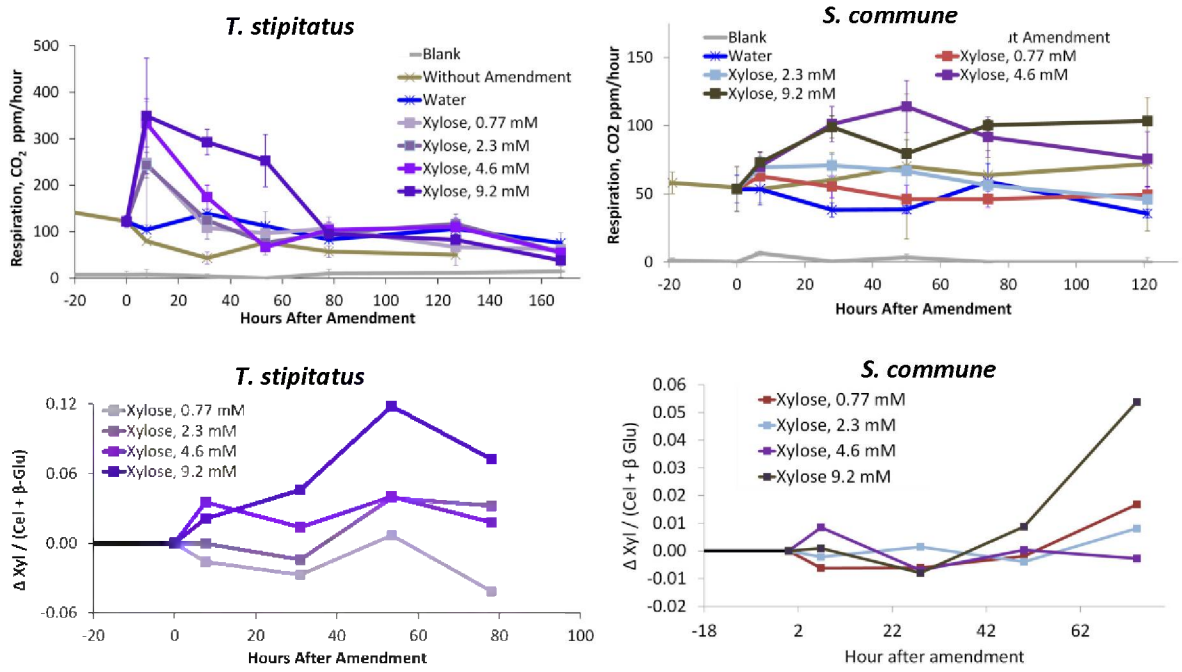
Opportunistic cheating depends on species having different responses to the availability of monomers. We conducted this experiment to examine differences between two fungi and quantify the speed with which gene expression would respond to a monomer amendment, which was important for calibration of stable isotope-labeling experiments.

We performed detailed analyses of physiology and gene transcription in the model decomposer fungi *Talaromyces stipitatus* and *Schizophyllum commune*, and found that variation between these

organisms is consistent with our model of cheating through catabolite repression. *T. stipitatus* is an ascomycete that readily degrades labile plant material but rapidly sporulates, whereas *S. commune* is a white rot basidiomycete capable of wood degradation. Both were grown in microcosms 7-10 days on cellulose, requiring investment in extracellular cellulolytic enzymes. After respiration stabilized, glucose and xylose (several concentrations) were added to replicate microcosms, with water as a control.

Responses of *S. commune* to monomer amendment during growth on cellulose were gradual but sustained beyond 3 days, whereas *T. stipitatus* response was more rapid and strong, but not persistent (Fig. 1). There was a negligible increase in *S. commune* respiration in response to the lower xylose concentrations (Fig. 1). For most monomer amendments, *T. stipitatus* respiration rate peaked only 6 hr after substrate amendment. Xylose only affected *S. commune* enzyme activities at the highest concentration of xylose (9.2 mM) more than 50 hrs after amendment. This is again in contrast with *T. stipitatus*, which had induced xylan-degrading enzyme activity within 6 hr of xylose amendment.

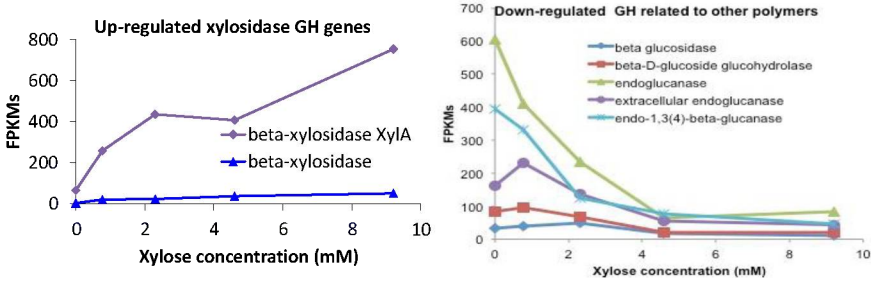
Fig. 1. Changes in respiration rate and enzyme activity of *T. stipitatus* and *S. commune* in response to increasing xylose amendments while growing on cellulose in sand microcosms.



We also observed dramatic shifts in *T. stipitatus* gene expression 6 hr after monomer amendment (Fig. 2). Illumina mRNA-seq was used to generate ~175 million paired-end sequencing reads from 36 samples. Over half of the glycosyl hydrolases (GHs) for which transcription was detected were significantly up- or down-regulated in response to increasing xylose amendment (33 of 61 genes; $P<0.05$). ***Up-regulated genes include both of the beta-xylosidases for which transcription was detected, whereas down-regulated genes include several glycosyl hydrolases involved in cellulose degradation (cellobiohydrolases, beta-glucosidases, and endoglucanases) (Fig. 2).*** Similarly, expression of over half of the major facilitator superfamily (MFS) transporter proteins were significantly up- or down-regulated (31 of 55 transcribed genes; $P<0.05$), and most of these were up-regulated. Interestingly, genes that were up-regulated (beta-xylosidases and most MFS transporters) tended to respond to the lowest concentration xylose amendment (0.77 mM), whereas genes that were down-regulated required a higher concentration of xylose (4.6 mM, generally) before significant down-regulation was observed.

The data described above demonstrate that *T. stipitatus* is adapted to rapidly responding to small changes in available monomer concentrations by increasing uptake and altering gene expression. *T. stipitatus* appears to respond more rapidly and at a lower monomer concentration than *S. commune.* Furthermore, *T. stipitatus* rapidly takes up and utilizes xylose at all concentrations, but xylan-degrading enzyme activity is only induced at the higher xylose concentrations. ***These traits should provide T. stipitatus the ability to opportunistically cheat when grown with S. commune.***

Fig. 2. *T. stipitatus* glycosyl hydrolase gene expression in response to increasing levels of xylose amendment while growing on cellulose.



2. Metabolism and cheating in mixed cultures of *T. stipitatus* and *S. commune*

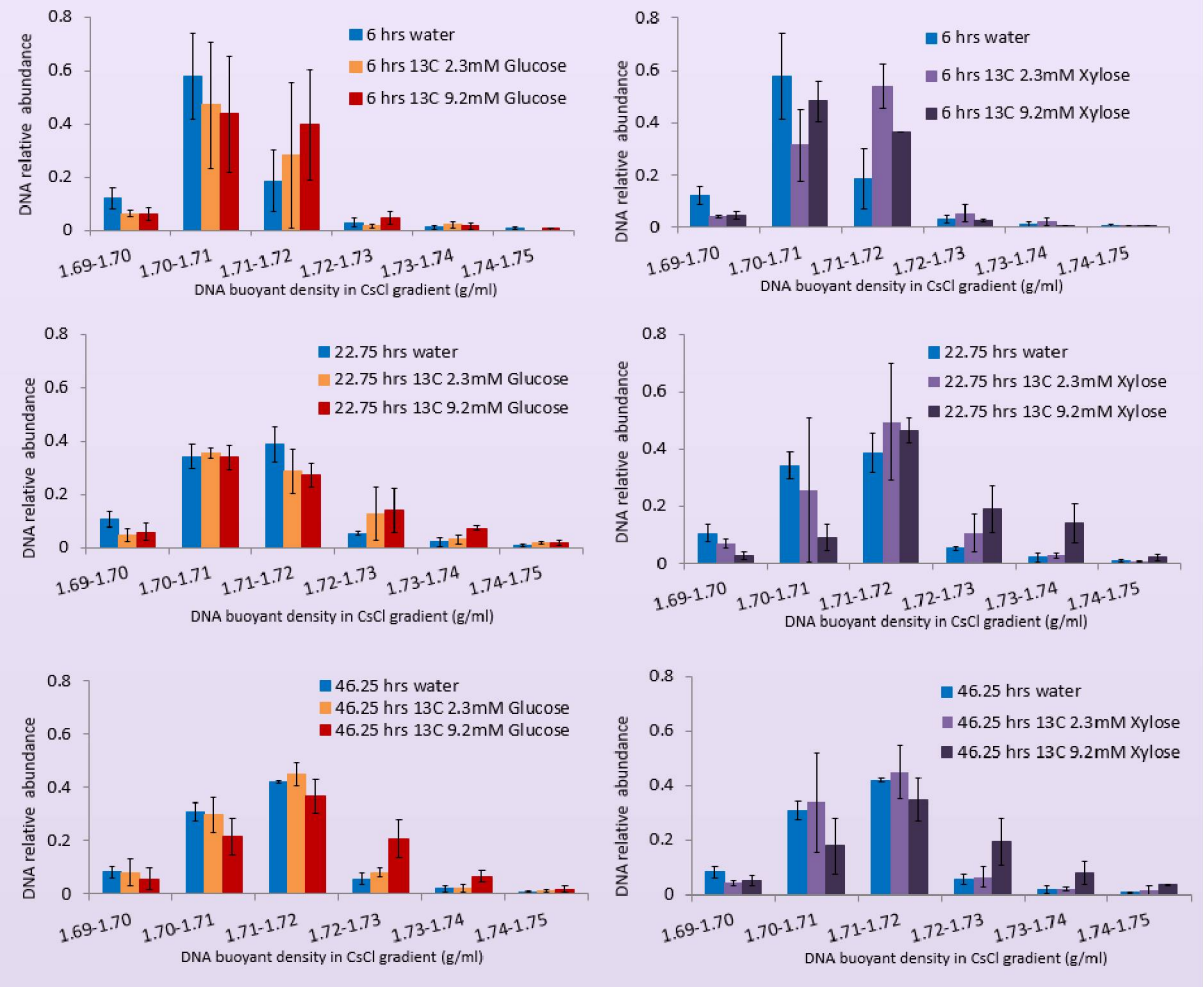
The prediction above has been tested using a mixed culture experiment in which *T. stipitatus* and *S. commune* were grown together in microcosms on cellulose, and then ^{13}C-labeled xylose and glucose were added at two concentrations (9.2 and 2.3 mM), with water amendment as a control. In this experiment, cheating can be inferred for an organism if it consumed a larger proportion of glucose than expected based on the proportion of cellulose-degrading ECE produced. Because the organisms are not growing on xylan, consumption of xylose should be low and is indicative that a species could switch to cheating in mixed culture. DNA SIP was used to detect uptake of ^{13}C-labeled monomers (Fig. 3). Metatranscriptome sequences have been obtained and are undergoing analysis to determine ECE production by each organism. Because this experiment used model organisms, we can directly determine the effects of growth in mixed culture on gene expression by comparison to transcriptomes from pure cultures described above.

Data collected from the ^{13}C-labeled treatments indicate that both glucose and xylose were rapidly consumed (within 6 hrs of amendment) at both monomer amendment concentrations. Maximum labeling of genomes was achieved 22.75 hrs after amendment and persisted to 46.25 hrs. Some genomes were almost fully labeled by ^{13}C (near buoyant density 1.75) (Fig. 3). Because of differences in buoyant density of unlabeled genomes, it is clear that *T. stipitatus* and *S. commune* were both consuming cellulose degradation products (the *T. stipitatus* peak merges with *S. commune* peak, which is also shifted to increased density). Xylose was also taken up and incorporated into DNA, but whether one or both species was involved is less clear because the unlabeled *T. stipitatus* peak persisted (Fig. 3). Shifang Hsu (postdoc) is finishing species-specific PCR on each density fraction to determine the amount carbon consumed by each organism.

3. Temperature, substrate complexity, and cheating in mixed cultures of *Trichoderma reesei*, *Phanerochaete chrysosporium*, and *Rhodotorula mucilaginosa*

We performed a similar mixed culture experiment to expand the range of species investigated and also investigate the impacts of temperature and substrate on cheating. The species used included *Rhodotorula graminis* (yeast basidiomycete), *Phanerochaete chrysosporium* (wood-rot basidiomycete), and *Trichoderma reesei* cellulose (rapid-growing ascomycete). These species differ vastly in genomic composition and physiological traits. *R. graminis* has few genes for ECE and grows very little on cellulose and pectin; thus, it is likely to be an obligate cheater. *T. reesei* and *P. chrysosporium* display

Fig. 3. Density distribution of DNA from mixed cultures (n=3) incubated after amendment with ^{13}C-labeled monomers. Unlabeled *T. stipitatus* DNA density is ~1.70; unlabeled *S. commune* DNA density is ~1.72. Controls amended with H_2O are shown for comparison. DNA abundance (Y-axis) is shown relative to the highest concentration density fraction of each sample.

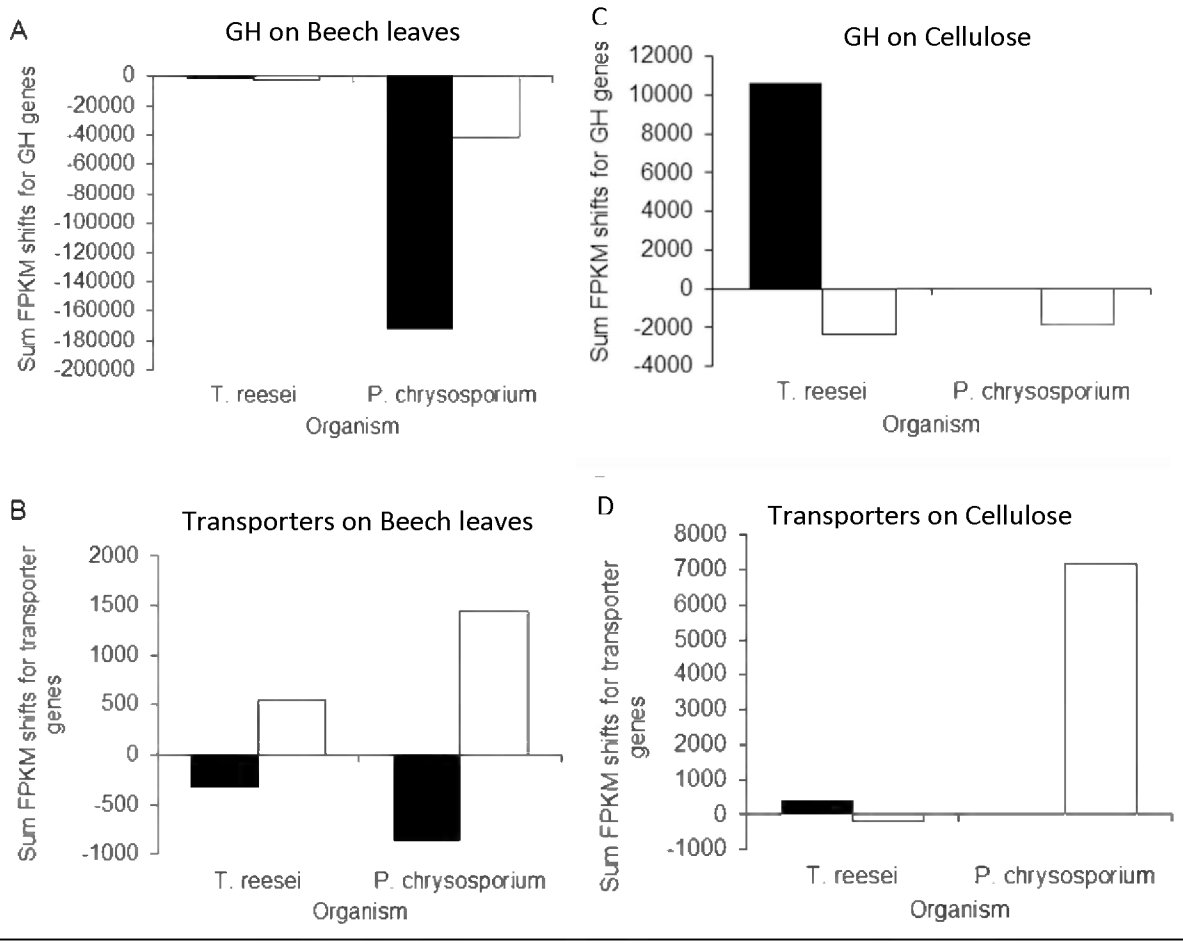


approximately equal growth and respiration on xylan and pectin, although *T. reesei* was able to utilize more cellulose. Although they have similar numbers of degradative genes and transporters, analysis of PFAM groups indicates that their complements of these genes are derived from different gene families and thus transcriptionally regulated by different mechanisms. Finally, *T. reesei* grows and sporulates more rapidly, and thus may be able to quickly respond to environmental perturbation and act as an opportunistic cheater. Thus, we expected *T. reesei* to act as the dominant decomposer during growth on cellulose and *P. chrysosporium* to be the dominant decomposer during growth on beech (*Fagus grandifolia*) leaves. Further we expected *R. mucilaginosa* to act as a cheater in all scenarios as it is not known to be a dominant degrader of cellulose or lignin.

However, when *P. chrysosporium* was grown in co-culture with *T. reesei*, *P. chrysosporium* down-regulated expression of glycoside hydrolase and peroxidase genes while up-regulating transporter genes, suggesting it adopted a cheating strategy, while growing on both cellulose and beech leaves (Fig. 4). This was the opposite of what we expected from *P. chrysosporium* because *P. chrysosporium* has the ability to degrade both lignin and cellulose. However, *T. reesei* is known to be a fast growing fungus that produces large quantities of cellulases. Its ability to grow rapidly suggests that *T. reesei* could take less

time to acclimatize to new growth conditions than *P. chrysosporium.* Hence, *T. reesei* may have begun to secrete cellulase enzymes first, which would catalyze hydrolysis of the cellulose fraction in beech leaves. Hence, the presence of these rapidly formed cellulose degradation products may have facilitated cheating by *P. chrysosporium.* In addition, *T. reesei* can be thought of as belonging to an early-colonizing guild that degrades available polymers, while *P. chrysosporium* belongs to the guild of lignin and polymer degraders. **Cheating may be a new mechanism by which some members of this slow growing, lignin-degrading guild can persist during dominance by organisms like *T. reesei*.** In this case, if all cellulose were hydrolyzed and taken up by the cells, *P. chrysosporium* could cease cheating and begin secreting lignin degrading enzymes.

Fig. 4. Shifts in glycoside hydrolase gene expression [A and C], and transporter gene expression [B and D] of *T. reesei* and *P. chrysosporium* in response to growth in co-culture. Shown is gene expression in coculture minus gene expression in monoculture for each organism.



Our study also revealed evidence of cheating by *R. mucilaginosa.* We observed that *R. mucilaginosa* increased in biomass during growth in co-culture with *P. chrysosporium.* Previous studies have shown *R. mucilaginosa* to be incapable of degrading cellulosic substrates, so we can infer that *R. mucilaginosa* is acting as an obligate cheater during growth in co-culture, and taking up monomers produced by the exoenzymes secreted by *P. chrysosporium.*

We hypothesized that increases in temperature could increase the importance of cheating because of increases in aqueous diffusion of exo-enxymes and degradation products, leading to more degradation products available for uptake. Consistent with this hypothesis, when grown in co-culture with *T. reesei*, *P. chrysosporium* had a larger increase in transporter gene expression at 30 °C than 20 °C,

suggesting that cheating by *P. chrysosporium* was greater at 30 °C than 20 °C (Fig. 4). Increasing the expression of transporter genes would allow *P. chrysosporium* to take advantage of the increased concentration of degradation products by uptake at a faster rate. We also found that temperature significantly reduced the carbon use efficiency, particularly when organisms were growing in co-culture. Hence, based on our results we expect cheating to be more prominent at higher temperatures. Devinda Hiriipitiyage (Master's student) and Mui Clark (PhD student) are finishing stable isotope probing work on this project to determine the amount carbon consumed by each organism.

4. Cheating can also directly affect plant nutrient uptake through interactions between decomposers and mycorrhizal fungi.

In addition to obtaining carbon from tree roots, ectomycorrhizal fungi may also compete with saprotrophs for litter or soil carbon when the supply from the plant is limited. Although EMF are not efficient plant litter degraders, and litter colonized by EMF alone is slow to decompose, EMF have access to a source of symbiotic plant carbon unavailable to saprotrophs, thus potentially inflating their population size relative to their saprotrophic ability. However, there is considerable debate surrounding the lignolytic and facultative saprotrophic behavior of EMF. Here we propose that during periods of low supply of photosynthate from a host plant, EMF may instead 'cheat', or capture labile substrates produced by ECE of saprotrophs.

To test this hypothesis across a range of EMF types, we performed microcosm experiments as described above using ground beech or sugar maple leaves as substrate. Organisms used included three EMF species (*Cenococcum geophilum*, *Laccaria bicolor*, and *Boletus edulis*) and two polymer degrading saprotrophs (*Phanerochaete chrysosporium* and *Talaromyces stipitatus*). Each of these species was grown in isolation as well as in pairs of one EMF species plus one saprotrophic species. After one week of growth, microcosms were harvested for physiological measurements and transcriptome sequencing, and DNA of organisms was labeled by amending microcosms with ^{13}C-enriched substrates.

Respiration of microcosms increased due to presence of the EMF, relative to the saprotrophic microbe monocultures. However, enzyme activities of mixed cultures primarily reflected values seen in saprotrophic organism monocultures, or were slightly reduced. Increased respiration without increasing enzyme activity suggests that one organism may be taking advantage of the activity of the other. Additional measurements of the abundance of each organism, consumption of specific monomers by each organism (SIP) and the sequencing of metatranscriptomic mRNA are underway by Mui Clark (Ph.D. student), and will permit us to discern which of these organisms is expressing the genes and will aid us in confirming this interpretation.

5. Bioinformatics tool development

Lack of development and testing of bioinformatics tools is one of the greatest challenges to making use of the new deluge of sequencing data (genomic, transcriptomic, metagenomics, metatranscriptomic) that is becoming available. To help deal with this issue, we developed software that will be useful for obtaining and organizing sequence data (Essinger et al. 2015) and finding relationships between taxonomic and functional gene aspects of sequence data and environmental variables and physiological capacities. New tools have been made available through KBase and other sites.

Sequences obtained from any project must be classified taxonomically and functionally by comparison to existing sequence databases. Although this has been optimized and extensively tested for bacterial small subunit ribosomal sequences, meta-omics methods creates the need for further development for functional genes and non-bacterial sequences. We developed and tested a variety of tools to improve identification of novel taxa and classification of metagenomics reads from diverse sources (e.g., Rosen and Essinger 2010, Rosen et al. 2011, Yok and Rosen 2011, Rosen et al. 2011, Lan et al. 2012, Rosen and Lim 2012).

Omics datasets are also characterized by high dimensionality – a very large number of descriptors relative to the number of objects or observations. Thus, we used machine learning approaches to develop and test feature selection tools that can extract major features of large (millions of reads) sequence datasets, or select the most informative features relative to a specific objective function (Garbarine et al. 2011, Xin et al. 2012, Ditzler et al. 2015a, Ditzler et al. 2015b, Ditzler et al. 2015c). Even more subtle signals exist in metagenomics datasets that can be structured simultaneously by multiple ecological phenomena, including selection by environmental gradients and dispersion of similar species by competitive interactions. To detect these patterns, we developed the first algorithms for combining redundancy analysis and co-occurrence analysis. This algorithm is in final testing by Steve Essinger (Ph.D.).

6. Prediction of microbial physiology from genome sequences

Newly sequenced fungal genomes have been used to infer possible ecological niches and traits of organisms, but these inferences have rarely been verified experimentally. When microorganism genomes are compared, distinct sets of ECE genes are revealed. This variability in genome content is often used to make predictions of metabolic responses of microorganisms under different environmental conditions. By monitoring the growth of 24 microorganisms with genome sequences available, cultured on four different polymer substrates, our objective in this project was to identify functional signatures in genomes allowing predictions of behavior on a particular substrate.

For this controlled microcosm experiment, microorganisms with available genome sequences were selected to establish whether gene copy numbers for extracellular enzymes can predict metabolic functional traits. The organisms selected represent a range of ecological niches, from polymer degraders such as *S. commune* to organisms that persist in the environment despite low degradation capability such as *Rhodoturula graminis*. We have found surprising results indicating that ECE activity is best predicted by copy numbers of unexpected gene families that allegedly correspond with alternative enzymatic functions (e.g., beta-glulcosidase activity is better predicted by nagase gene copy number than beta-glucosidase gene copy number). This indicates either that there is a more complicated relationship than expected between gene content of a genome and a specific function, or that current functional characterizations of gene families (at least, glycosyl hydrolase families) is incomplete. This project is in final stages of analysis by Nivedita Clark (Ph.D. student).

7. Forest Root Biomass Mediated by Tree Root Interactions.

Most organic matter stored in forest soil is derived from root biomass. A new area of research involves examination of drivers of root biomass variation. This involves understanding the structure of root traits across plant species, and whether it is predictable from environmental variables, plant identity, aboveground plant traits, or other characteristics. Because root litter is the major form stored in soil and the major site of plant-microbe interactions, these investigations are critical for understanding how the microbial community and physiological ecology described above will translate to the field.

We used a molecular approach borrowed from microbial ecology to identify roots in mixed-species temperate forests. We found that 75% of root traits were phylogenetically structured, as opposed to only 28% of aboveground traits (Valverde-Barrantes et al. 2015a). Basal angiosperm lineages had thicker, less branched roots with higher N and lower lignin content compared to more evolutionary derived lineages. Root nitrogen was the only trait correlated with an analogous leaf trait (Valverde-Barrantes et al. 2015a). Furthermore, these phylogenetically structured root traits were closely related to the extent of colonization by arbuscular mycorrhizal fungi (Valverde-Barrantes et al. 2016), a primary mechanism by which plants obtain nutrients and contribute carbon to the soil.

We then used molecular barcoding to identify interacting roots coexisting in a natural mixed-species forest community. In contrast to expectations based on niche partitioning, we found that

biomass increased for all species in response to soil resources, and there was little vertical or horizontal spatial segregation among species (Valverde-Barrantes et al. 2015b). A positive relationship between root phylogenetic diversity and fine root biomass indicated significant trait-mediated overyielding, possibly caused by plant-pathogen interactions. These results suggest that fine root biomass is affected by symmetric responses to soil properties coupled with complementary species traits. Current research is investigating the consequences of this structure in root traits for plant-pathogen interactions and decomposer soil microbial communities and carbon sequestration.

8. Pectinase primer development

Currently, there are PCR primer sets appropriate for use in a natural community setting for two fungal extracellular enzymes: laccase, targeting lignin, and cellobiohydrolase, targeting cellulose. To augment the number of extracellular enzyme gene assays at our disposal, we developed PCR primers for fungal pectinase genes (Gacura et al. 2016). We targeted a clade of fungal glycosyl hydrolase family 28 that includes known fungal endopolygalacturonases.

PCR amplification using our experimental primers was performed on DNA extractions from individual leaves of dominant tree species in different ecosystems in Manistee National Forest. These ecosystem types are well known to harbor microbial communities with differing taxonomic composition. Unexpectedly, however, pectinase composition was more affected by the particular forest stand sampled than by ecosystem differences. Environmental similarity of forest stands within an ecosystem type implies that populations may be differentiated in pectinase OTU composition due to drift resulting from dispersal limitation and neutral dynamics, rather than adaptations to environmental conditions.

9. Phytobeneficial bacteria in Nigeria maize production

A visiting scholar from the University of Ibadan, Nigeria, visited our lab to identify bacteria isolated from the rhizosphere of maize growing in southwestern Nigeria. Unlike most proposed microbial-based biofertilizers, these strains were isolated from Nigerian maize fields and were shown to benefit maize growth in *in situ* in Nigerian trials (Abiala et al. 2015). Molecular analysis showed that the strains were related to the genera *Bacillus* (isolate EBS8), *Citrobacter* (ADS14), *Enterobacter* (IGBR11), and *Lysinibacillus* (EPR2) (Abiala et al. 2015). They show great promise both for the control of maize diseases caused by *Fusarium verticillioides* and for the improvement of maize nutrition, and field testing is now underway.

Grant Products

Journal articles published

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Journal articles in preparation

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